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(54) Title: FERMENTED TEA BEVERAGE AND TEA BEVERAGE

(54) 発明の名称: 茶－発酵飲料および茶飲料

(57) Abstract: A fermented tea beverage containing a fermented tea solution produced with at least one lactic acid bacterium selected from the group consisting of *Lactobacillus* ONRIC b0239 (FERM BP-10064) and *Lactobacillus* ONRIC b0240 (FERM BP-10065); and a tea beverage containing the lactic acid bacterium.

(57) 要約: 本発明は、ラクトバチルス ONRIC b0239(FERM BP-10064)およびラクトバチルス ONRIC b0240(FERM BP-10065)からなる群から選択される少なくとも１種の乳酸菌の茶発酵液を含む茶－発酵飲料および、該乳酸菌を含む茶飲料を提供する。

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明 細 書

茶－発酵飲料および茶飲料

技術分野

[0001] 本発明は、茶－発酵飲料および茶飲料に関する。

背景技術

[0002] 従来、以下の事項が知られている。

[0003] (1)伝統的に茶葉を発酵させることは知られており、碁石茶、プーアル茶、ミアン茶などの発酵茶が知られている。

[0004] (2)茶葉から茶葉中で増殖し得るラクトバチルス属プランタラム種菌(乳酸菌)を分離して液体培地で培養し、茶葉を熱湯に浸漬した後放冷し、この放冷物に前記培養菌を接種して所定期間発酵させてなる緑茶発酵物が知られている(特許文献1)。

[0005] (3)酵母及び細菌を用いて、茶またはコーヒーから、簡単な製造条件および比較的短い製造時間で大量の醗酵飲料を製造する方法が知られている(特許文献2)。

[0006] (4)粘膜免疫は、粘膜上に病原体が付着した際の最初に行われる感染防御機構である(非特許文献1)。

[0007] (5)粘液中の分泌型IgA(S－IgA)は、バクテリア、ウイルスなどの病原体に対する防御作用を示す(非特許文献2および3)とともに、微生物の産生する毒素を中和する役割も担っている(非特許文献4)。

特許文献1:特許2876006号

特許文献2:特開平9－220054号

非特許文献1:Brandtzaeg, P. Curr. Top. Microbiol. Immunol. 146:13 1989

非特許文献2:Czinn, S, J. et al., Vaccine 11:637 1993

非特許文献3:Renegar, K. et al., J. Immunol. 146:1972 1991

非特許文献4:Brandtzaeg, P APMIS 103:1 1995; Kilian, M. et al Microbiol. Rev. 52: 296 1988

発明の開示

発明が解決しようとする課題

[0008] 本発明はラクトバチルス属プランタラム種菌(乳酸菌)を用いて発酵させた茶、もしくは該菌を添加した茶であって、従来知られていない高いIgA産生亢進作用を有する飲料を提供することを目的とする。

課題を解決するための手段

[0009] 本発明者らは、先に優れた粘膜免疫賦活作用、生体防御機構向上作用などを奏し得、プロバイオティックスとして有用な新しい2種の乳酸菌、ラクトバチルス ONRIC b0239 (FERM BP-10064)およびラクトバチルス ONRIC b0240 (FERM BP-10065)を新たに見出し、この乳酸菌に係る発明を特許出願した(特願2003－297570号、PCT／JP2004／012136)。

[0010] 本発明者らは、鋭意研究の結果、先に見出した新規な乳酸菌は、これを茶抽出液に配合する場合に、お茶本来の風味などに悪影響を与えず、しかも上記乳酸菌の有する優れたIgA産生亢進作用または粘膜免疫賦活作用は保持されるという事実を見出した。また、該乳酸菌は茶抽出液中でも発酵(培養、増殖)容易であり、しかも、かくして得られる茶－発酵飲料は、お茶本来の風味などに悪影響を受けておらず、該乳酸菌に由来する優れたIgA産生亢進作用または粘膜免疫賦活作用を奏し得るという事実を見出した。本発明は、これらの知見を基礎として更に研究を重ねた結果、完成されたものである。

[0011] 本発明は、下記項1～23に記載の要旨の発明を提供する。

[0012] 項1. ラクトバチルス ONRIC b0239 (FERM BP-10064)およびラクトバチルス ONRIC b0240 (FERM BP-10065)からなる群から選択される少なくとも1種の乳酸菌の茶発酵液を含有することを特徴とする茶－発酵飲料。

[0013] 項2. 更に茶抽出液を含む項1に記載の茶－発酵飲料。

[0014] 項3. 乳酸菌を、粘膜免疫賦活作用を発揮する有効量含有する項1または項2に記載の茶－発酵飲料。

[0015] 項4. 乳酸菌を、IgA産生亢進作用を発揮する有効量含有する項1または項2に記載の茶－発酵飲料。

[0016] 項5. 乳酸菌を、茶－発酵飲料中に10^4cfu／mL～10^8cfu／mL含有する項1または項2に記載の茶－発酵飲料。

[0017] 項6. 乳酸菌を、茶－発酵飲料中に10^5cfu／mL～10^7cfu／mL含有する項1または項2に記載の茶－発酵飲料。

[0018] 項7. ラクトバチルス ONRIC b0239 (FERM BP-10064)およびラクトバチルス ONRIC b0240 (FERM BP-10065)からなる群から選択される少なくとも1種の乳酸菌および茶抽出液を含有することを特徴とする茶飲料。

[0019] 項8. 乳酸菌を、粘膜免疫賦活作用を発揮する有効量含有する項7に記載の茶飲料。

[0020] 項9. 乳酸菌を、IgA産生亢進作用を発揮する有効量含有する項7に記載の茶飲料。

[0021] 項10. 乳酸菌を、茶飲料中に10^4cfu／mL～10^8cfu／mL含有する項7に記載の茶飲料。

[0022] 項11. 乳酸菌を、茶飲料中に10^5cfu／mL～10^7cfu／mL含有する項7に記載の茶飲料。

[0023] 項12. 茶含有培地でラクトバチルス ONRIC b0239 (FERM BP-10064)およびラクトバチルス ONRIC b0240 (FERM BP-10065)からなる群から選択される少なくとも1種の乳酸菌を培養する工程を含む、項1に記載の茶－発酵飲料の製造方法。

[0024] 項13. 茶－発酵飲料の乳酸菌含有量を、10^4cfu／mL～10^8cfu／mLに調整する工程を更に含む、項12に記載の方法。

[0025] 項14. 茶含有培地が、任意成分を含んでいてもよい、茶由来のブリックス度0. 10～0. 50の茶抽出液であり且つ培養が、25℃～50℃の温度で12時間～32時間を要して行われる項12または項13に記載の製造方法。

[0026] 項15. 茶含有培地が、任意成分を含んでいてもよい、茶由来のブリックス度0. 18～0. 30の茶抽出液であり且つ培養が、30℃～40℃の温度で15時間～20時間を要して行われる項12または項13に記載の製造方法。

[0027] 項16. 項2に記載の茶－発酵飲料の製造方法であって、下記の工程：

(1)茶含有培地でラクトバチルス ONRIC b0239 (FERM BP-10064)およびラクトバチルス ONRIC b0240 (FERM BP-10065)からなる群から選択される少なくとも1種の乳酸菌を培養して茶発酵液を得る工程、

および、

(2)上記(1)の工程によって得られた茶発酵液に茶抽出液を添加する工程、

を含む方法。

[0028] 項17. (2)の工程において、茶抽出液を、最終的な茶－発酵飲料の茶由来のブリックス度が0. 10～0. 50となり、且つ乳酸菌含有量が10^4cfu／mL～10^8cfu／mLとなるように茶発酵液に添加する、項16に記載の方法。

[0029] 項18. 項7に記載の茶飲料の製法であって、ラクトバチルス ONRIC b0239 (FERM BP-10064)およびラクトバチルス ONRIC b0240 (FERM BP-10065)からなる群から選択される少なくとも1種の乳酸菌と茶抽出液とを混合する工程を含む茶飲料の製造方法。

[0030] 項19. 茶抽出液を、最終的な茶飲料の茶由来のブリックス度が0. 10～0. 50となり、且つ乳酸菌含有量が10^4cfu／mL～10^8cfu／mLとなるように乳酸菌と混合する、項18に記載の方法。

[0031] 項20. 粘膜免疫賦活作用を有する茶－発酵飲料の製造のための、ラクトバチルス ONRIC b0239 (FERM BP-10064)およびラクトバチルス ONRIC b0240 (FERM BP-10065)からなる群から選択される少なくとも1種の乳酸菌の使用。

[0032] 項21. IgA産生亢進作用を有する茶－発酵飲料の製造のための、ラクトバチルス ONRIC b0239 (FERM BP-10064)およびラクトバチルス ONRIC b0240 (FERM BP-10065)からなる群から選択される少なくとも1種の乳酸菌の使用。

[0033] 項22. 粘膜免疫賦活作用を有する茶飲料の製造のための、ラクトバチルス ONRIC b0239 (FERM BP-10064)およびラクトバチルス ONRIC b0240 (FERM BP-10065)からなる群から選択される少なくとも1種の乳酸菌の使用。

[0034] 項23. IgA産生亢進作用を有する茶飲料の製造のための、ラクトバチルス ONRIC b0239 (FERM BP-10064)およびラクトバチルス ONRIC b0240 (FERM BP-10065)からなる群から選択される少なくとも1種の乳酸菌の使用。

[0035] 本発明の茶－発酵飲料および茶飲料は、従来のお茶の味を大切に温存させたものである。特に、本発明の茶－発酵飲料は発酵させているにも拘わらず、発酵臭および従来の発酵茶などに特有の味を有しないかもしくは軽微にしたものである。本発

明の茶－発酵飲料および茶飲料は、IgA産生亢進作用および粘膜免疫賦活作用を奏し得ることを特徴としている。

[0036] 以下、本発明の茶－発酵飲料および茶飲料に利用する特定の乳酸菌につき詳述し、次いで、本発明の茶－発酵飲料および茶飲料につき詳述する。

[0037] 乳酸菌

本発明の茶－発酵飲料および茶飲料に利用する乳酸菌は、先に本発明者らが新たに天然物から以下の通り分離・採取（スクリーニング）し且つ寄託したものである。それぞれ、ラクトバチルス（Lactobacillus）ONRIC b0239（FERM BP-10064)およびラクトバチルス（Lactobacillus）ONRIC b0240（FERM BP-10065)と命名される(以下、両者を、「ONRIC乳酸菌」と総称する場合がある。)。

[0038] （1）スクリーニング

（1－1）起源微生物

起源微生物としては、ヒト腸内容物、植物性食品および動物性食品から分離され、大塚製薬株式会社大津栄養製品研究所で保存している乳酸菌を利用する。

[0039] （1－2）スクリーニング方法

目的とする乳酸菌のスクリーニングは、マウスパイエル板細胞培養系を用いて、IgA産生誘導能を指標として実施する。該IgA産生能の試験方法の詳細は、後記試験例1に示すとおりである。

[0040] （2）スクリーニングされた微生物

（2－1）ラクトバチルス ONRIC b0239

（a）肉眼的特徴

（a－1）MRS寒天培地

円形からやや不規則、半球形、平滑、乳白色

（a－2）BL寒天培地

円形からやや不規則、半球形、平滑、白褐色

（b）顕微鏡的特徴

桿菌で運動性を持たない。芽胞は形成しない。

[0041] （c）生育温度

30～33℃で良好に発育する。

[0042] (d)生理学的、生化学的特徴

グラム染色性:陽性

糖資化性

Glycerol	−
Erythritol	−
D-Arabinose	−
L-Arabinose	−
Ribose	±
D-Xylose	±
L-Xylose	−
Adonitol	−
β-Methyl-D-Xyloside	−
Galactose	+
D-Glucose	+
D-Fructose	+
D-Mannose	+
L-Sorbose	−
Rhamnose	−
Dulcitol	−
Inositol	−
Mannitol	−
Sorbitol	+
α-Methyl-D-Mannoside	+
α-Methyl-D-Glucoside	±
N-Acetyl-Glucosamine	+
Amygdalin	+
Arbutin	+
Esculin	+
Salicin	+
Cellobiose	+
Maltose	+
Lactose	+
Melibiose	+
Saccharose	+
Trehalose	+
Inulin	−

Melezitose	−
D-Raffinose	+
Amidon	−
Glycogen	−
Xylitol	−
β-Gentiobiose	+
D-Turanose	−
D-Lyxose	−
D-Tagatose	−
D-Fucose	−
L-Fucose	−
D-Arabitol	±
L-Arabitol	−
Gluconate	−
2-Keto-Gluconate	−
5-Keto-Gluconate	−

以上の諸性質から、バージィーズ・マニュアル・オブ・システマティック・バクテリオロジー(Bergey's Manual of Systematic Bacteriology)に照らし、本菌株をLactobacillus plantarumに属する菌株と同定し、Lactobacillus ONRIC b0239と命名し、平成15年8月6日に、日本国茨城県つくば市東1－1－1　中央第6に住所を有する独立行政法人　産業技術総合研究所　特許生物寄託センター(AIST)に寄託番号FERM P-19469として寄託した。該微生物は、現在、国際寄託に移管されており、その国際寄託番号はFERM BP-10064である。

[0043] (2－2)ラクトバチルス ONRIC b0240

(a)肉眼的特徴

(a－1)MRS寒天培地

円形からやや不規則、半球形、平滑、乳白色

(a－2)BL寒天培地

円形からやや不規則、半球形、平滑、白褐色

(b)顕微鏡的特徴

桿菌で運動性を持たない。芽胞は形成しない。

[0044] (c)生育温度

30～33℃で良好に発育する。

[0045] (d)生理学的、生化学的特徴

グラム染色性:陽性

糖資化性

Glycerol	−
Erythritol	−
D-Arabinose	−
L-Arabinose	−
Ribose	±
D-Xylose	−
L-Xylose	−
Adonitol	−
β-Methyl-D-Xyloside	−
Galactose	+
D-Glucose	+
D-Fructose	+
D-Mannose	+
L-Sorbose	−
Rhamnose	−
Dulcitol	±
Inositol	−
Mannitol	+
Sorbitol	+
α-Methyl-D-Mannoside	−
α-Methyl-D-Glucoside	−
N-Acetyl-Glucosamine	+
Amygdalin	+
Arbutin	+
Esculin	+
Salicin	+
Cellobiose	+
Maltose	+
Lactose	+
Melibiose	+
Saccharose	+
Trehalose	−
Inulin	−
Melezitose	−
D-Raffinose	+
Amidon	−
Glycogen	−

Xylitol	−
β-Gentiobiose	+
D-Turanose	−
D-Lyxose	−
D-Tagatose	−
D-Fucose	−
L-Fucose	−
D-Arabitol	−
L-Arabitol	−
Gluconate	−
2-Keto-Gluconate	−
5-Keto-Gluconate	−

以上の諸性質から、バージィーズ・マニュアル・オブ・システマティック・バクテリオロジー(Bergey's Manual of Systematic Bacteriology)に照らし、本菌株をLactobacillus plantarumに属する菌株と同定し、Lactobacillus ONRIC b0240と命名し、平成15年8月6日に、日本国茨城県つくば市東1－1－1 中央第6に住所を有する独立行政法人 産業技術総合研究所 特許生物寄託センター(AIST)に寄託番号FERM P-19470として寄託した。該微生物は、現在、国際寄託に移管されており、その国際寄託番号はFERM BP-10065である。

[0046] ONRIC乳酸菌に認められる特有の粘膜免疫賦活作用およびこれに寄与するIgA産生亢進作用は、次のように考えられている。即ち、まず腸管免疫系を構成するパイエル板のM細胞が管腔にある抗原を取り込む。該抗原は樹状細胞などの抗原提示細胞によってCD4 T細胞に提示される。抗原特異的なT細胞の作用により、未熟なB細胞が成熟しつつ粘膜固有層に移動して最終的にIgA抗体産生細胞に分化する。このIgA産生亢進機構にONRIC乳酸菌がどのように関与するかについては現在なお明確ではないが、少なくともONRIC乳酸菌の存在によってIgA産生亢進がなされるためにはパイエル板のM細胞が抗原を取り込む必要があることから、ONRIC乳酸菌はこの抗原としての機能を果たし得るものであると考えられる。この抗原としての機能を果たすという面からは、ONRIC乳酸菌は、特に生菌である必要はなく、通常の一般的加熱滅菌操作によって滅菌されたものであってもよい。

[0047] 茶－発酵飲料

本発明の茶ー発酵飲料は、ラクトバチルス ONRIC b0239 (FERM BP-10064)およびラクトバチルス ONRIC b0240 (FERM BP-10065)からなる群から選択される少なくとも1種の乳酸菌(ONRIC乳酸菌)の茶発酵液をその必須成分として含有することを特徴とする。必須成分としての茶発酵液は、茶を含有する培地でONRIC乳酸菌を培養することにより調製される。本発明の茶発酵液には、培養後のONRIC乳酸菌を含む。本発明において、ONRIC乳酸菌を含む培養後の茶含有培地は、そのまま本発明の茶発酵液とすることができる。

[0048] 本明細書において、茶含有培地とは、適当な溶媒(好ましくは水または湯)に茶葉および/またはその粉砕物(粉末など)を含むものであっても、茶葉および/またはその粉砕物を、適当な溶媒(好ましくは水または湯)で抽出して得られる茶抽出液であってもよく、また、該茶抽出液を噴霧乾燥手段などにより粉末化して得られる粉末(粉末エキス)を、適当な溶媒(好ましくは水または湯)に含むものであってもよい。これらの培地の原料として利用することができる茶(茶葉、Tea Sinensis)には、例えば、煎茶、玉露、棒茶、粉茶、番茶、ほうじ茶、玄米茶、抹茶などに分類される各種の緑茶類を代表として、他に、紅茶、烏龍茶、黒茶(プーアル茶、ミアン茶、碁石茶など)や、それら以外の植物からの茶様飲料であるハーブティー、ルイボス茶、甜茶、甘茶などが含まれる。これらは、一種単独で用いてもよく、二種以上を組み合わせて用いてもよい。

[0049] このような茶として具体的には、例えば、鉄観音茶、色種茶、水仙茶、黄金桂茶、煎茶、かぶせ茶などを挙げることができる。

[0050] 茶含有培地として、上記各種の茶葉および/またはその粉砕物を含む水または湯などを用いる場合、培地中に含まれる茶葉および/またはその粉砕物の量は、特に限定されるものではないが、一般には最終的な培地における茶由来のブリックス度が0.10～0.50、好ましくは0.18～0.30となるような範囲から選択することができる。

[0051] 「ブリックス度」とは、一般に溶液100gあたりの可溶性固形物重量(g)を表し、示差濃度計(例えば、デジタル示差濃度計 DD－5:株式会社アタゴ製)、屈折計などにより計測される。ブリックス度は、溶液中に複数の可溶性固形物が溶解している場合、それらの合計値として測定されるが、本明細書において、「茶由来のブリックス度」と

は、茶由来の可溶性固形物の濃度に基づいて測定されるブリックス度を表す。即ち、「茶由来のブリックス度」は、茶含有培地、茶抽出液、最終的な飲料などの溶液100gあたりに含まれる茶由来の可溶性固形物重量(g)を表す。

[0052] 茶含有培地として、上記各種の茶葉および／またはその粉砕物の抽出液を用いる場合は、一般的に飲用されるお茶と同様に、これらの茶葉などを水または湯で抽出したものを用いることができる。該抽出液の濃度は特に限定されるものではないが、通常、茶由来のブリックス度が0．10～0．50、好ましくは0．18～0．30となる範囲から選択される。

[0053] また、茶を含有する培地として、上記各種の茶葉を原料とする粉末エキスを含む水または湯などを用いる場合、粉末エキスとして具体的には、例えば、FD緑茶エキスパウダー、FDジャスミン茶エキスパウダー、FD烏龍茶エキスパウダー(以上、三栄源社)、麦茶エキストラクトパウダー、紅茶エキストラクトパウダー、緑茶エキストラクトパウダー(以上、高砂香料工業株式会社)などをあげることができる。これらの粉末エキスは、一般にこれらのうちの一種単独もしくは二種以上を水または湯に溶解した水溶液の形態で培地として利用することができる。これらの粉末エキスの水または湯への添加量は、特に限定されるものではないが、通常、得られる水溶液の茶由来のブリックス度が0．10～0．50、好ましくは0．18～0．30の範囲となる量から選択される。

[0054] 本発明の茶発酵液は、例えば、上記茶を含有する培地にONRIC乳酸菌を10^4～10^8cfu／mL(培地)となるように接種し、25～50℃の温度で、12時間～32時間培養することにより得ることができる。

[0055] ここで、ONRIC乳酸菌の培養(発酵)は、予め適当な発酵用培地に該乳酸菌を接種して培養したスターターを利用する方法によるのが好ましい。ここでスターターとしては、例えば代表的には予め90～121℃、5～20分間通常の殺菌処理を行った発酵用培地、例えばMRS培地、10%脱脂粉乳培地などに、凍結保存菌体、凍結乾燥菌体などの形態のONRIC乳酸菌を接種して培養したものを挙げることができる。このようにして得られるスターターは、通常、ONRIC乳酸菌を10^4～10^7cfu／g培養物程度含んでいるのが好ましい。

[0056] スターターとして用いられる発酵用培地には、必要に応じてONRIC乳酸菌の良好

な生育を行うための発酵促進物質、例えばグルコース、澱粉、蔗糖、乳糖、デキストリン、ソルビトール、フラクトースなどの炭素源、ペプトンなどの窒素源、ビタミン類、ミネラル類などを加えることができる。

[0057] スターターの調製および茶発酵液の調製におけるONRIC乳酸菌の培養条件は、一般に、発酵温度25～50℃程度、好ましくは30～40℃程度、発酵時間12～32時間程度、好ましく15時間～20時間程度から選ばれる。特に好ましい培養条件としては、33℃、16時間をあげることができる。

[0058] ONRIC乳酸菌の培養(発酵)の際には、更に必要に応じて、茶含有培地中に、該乳酸菌の維持、増殖などに適した栄養成分などの任意成分を適量含有させることもできる。該栄養成分の具体例としては、各微生物の培養のための培養培地に利用される例えばグルコース、澱粉、蔗糖、乳糖、デキストリン、ソルビトール、フラクトースなどの炭素源、例えばペプトンなどの窒素源、ビタミン類、ミネラル類、微量金属元素、その他の栄養成分などの各成分を挙げることができる。ビタミン類としては、例えばビタミンB、ビタミンD、ビタミンC、ビタミンE、ビタミンKなどを例示できる。微量金属元素としては、例えば亜鉛、セレンなどを例示できる。その他の栄養成分としては、例えば乳果オリゴ糖、大豆オリゴ糖、ラクチュロース、ラクチトール、フラクトオリゴ糖、ガラクトオリゴ糖などの各種オリゴ糖を例示できる。これらのオリゴ糖の配合量は、特に限定されるものではないが、通常、得られる茶発酵液中に3重量%程度以下となる量範囲から選ばれるのが好ましい。

[0059] かくして得られる茶発酵液は、そのまま本発明の茶－発酵飲料とすることができ、また、該茶発酵液を凍結乾燥などして得られる粉末エキスを水などに溶解して本発明の茶－発酵飲料とすることもできる。さらに、上記の如くして得られる茶発酵液を濃縮、または水などで希釈して本発明の茶－発酵飲料としてもよく、茶発酵液にさらに茶抽出液を加えて本発明の茶－発酵飲料としてもよい。ここで、茶発酵液に添加される茶抽出液は、上記の茶含有培地として使用される茶葉および／またはその粉砕物を適当な溶媒(好ましくは水または湯)で抽出して得られる茶抽出液と同様であり、また、該茶抽出液を噴霧乾燥手段などにより粉末化して得られる粉末(粉末エキス)を、水または湯などに溶解したものを用いることもでき、その濃度は特に制限されない。

[0060] 本発明茶－発酵飲料中のONRIC乳酸菌の含有量は、茶発酵液を利用する場合もその凍結乾燥品などを利用する場合もいずれも、一般には、10^4～10^8cfu／mL前後、好ましくは10^5～10^7cfu／mL前後となる量から適宜選択することができる。該乳酸菌の含有量は、茶含有培地の培養条件、菌の接種量などを変えることによって調整してもよく、茶発酵液を濃縮または希釈することにより調整してもよい。また、茶発酵液の粉末エキスを用いる場合には、該粉末エキスの添加量を変えることによってONRIC乳酸菌の含有量を調整してもよい。

[0061] 茶発酵液に茶抽出液を加えて本発明の茶－発酵飲料とする場合、両者の配合割合は、例えば、両者を混合して得られる本発明茶－発酵飲料が、該乳酸菌を10^4cfu／mL～10^8cfu／mL、好ましくは10^5cfu／mL～10^7cfu／mL含み且つ該茶－発酵飲料の茶由来のブリックス度が0．10～0．50、好ましくは0．18～0．30の範囲となるように適宜決定することができるが、これに限定されない。

[0062] ここで、cfu（コロニー形成単位）は下記方法により計測された生菌数で示されるものであって、実際に飲料中にこの数の生菌が含まれることを意味するものではない。即ち、本発明飲料は、一般には通常の加熱手段などによって殺菌されて製品とされるものであるため、該製品中には生菌は存在しない場合がある。そのような場合でも、該製品は殺菌前に計測された生菌数に相当する死菌が含まれており、本発明所期の効果を奏し得る。本明細書において、このような殺菌処理などを施された飲料についての菌数は、殺菌処理などを施す前に計測した生菌数にて示すものとする。

[0063] ＜乳酸菌数の計測＞

生菌数の測定は、BCP加プレートカウント寒天培地を用いて33℃下で混釈培養を行い、生育したコロニー数を計測することにより求められる。この生菌数と濁度とは相関するため、予め生菌数と濁度との相関を求めておくと、生菌数の測定に代えて濁度を測定することによって上記生菌数を計数できる。

[0064] 本発明茶－発酵飲料中へのONRIC乳酸菌の配合量は、上記菌数を目安として、調製される飲料の種類、利用する乳酸菌の種類などに応じて適宜変更することができる。

[0065] 本発明茶－発酵飲料には、更に必要に応じて、通常の飲食品と同様に、適当な可

食性担体(食品素材)を添加することができる。

[0066] かくして、本発明の茶－発酵飲料を得ることができる。本発明の茶－発酵飲料は、最終的に適当な容器に無菌的に充填して飲料製品とすることができる。この製品は、お茶本来の風味を有しているのに加えて、含有するONRIC乳酸菌に由来する特有の粘膜免疫賦活作用またはIgA産生亢進作用を有している。

[0067] 本発明茶－発酵飲料の摂取量は、これを摂取する生体の年齢、性別、体重、疾患の程度などに応じて適宜決定され、特に限定されるものではないが、一般には、該飲料中に含まれるONRIC乳酸菌数に換算して、1日ヒト1人当たりの乳酸菌摂取量(菌数)が約10^6～10^{10}cfuとなる範囲から選ばれるのがよい。従って、本発明茶－発酵飲料の摂取量はまた、該飲料中の菌数に応じて適宜選択され得るが、一般には約50～1000mLの範囲から選択されるのが好ましい。

[0068] 本発明はまた、粘膜免疫賦活作用またはIgA産生亢進作用を有する茶－発酵飲料の製造のための、ラクトバチルス ONRIC b0239 (FERM BP-10064)およびラクトバチルス ONRIC b0240 (FERM BP-10065)からなる群から選択される少なくとも1種の乳酸菌の使用をも提供する。

[0069] 茶飲料

本発明は、ラクトバチルス ONRIC b0239 (FERM BP-10064)およびラクトバチルス ONRIC b0240 (FERM BP-10065)からなる群から選択される少なくとも1種の乳酸菌(ONRIC乳酸菌)を含む茶飲料をも提供する。この茶飲料は、該乳酸菌と茶抽出液とを必須成分として含有する。

[0070] ここで、利用されるONRIC乳酸菌は、前述した本発明茶－発酵飲料の調製に利用される茶発酵液から単離して得られる菌体またはその凍結乾燥品などであってもよい。また、本発明の茶飲料に利用される乳酸菌は、ONRIC乳酸菌を、茶を含有しない適当な培地で培養して得られる、該乳酸菌を含む培養液の形態であってもよく、また、該培養液から単離して得られる菌体またはその凍結乾燥品などの形態であってもよい。

[0071] 茶を含有しない培地としては、前述したMRS培地、10%脱脂粉乳培地などの、通常の乳酸菌の培養に用いられることの知られている栄養培地の他、例えば野菜類、

果実類、豆乳(大豆乳化液)などを発酵用原料物質として含む液をあげることができる。

[0072] 本発明茶飲料の製造に利用される乳酸菌は、これらの液中でONRIC乳酸菌を培養して得られる発酵液(培養液)の形態であってもよく、該発酵液を利用して得られる粗精製品、精製品、これらの凍結乾燥粉末などであってもよい。

[0073] 発酵用原料物質として利用される野菜類および果実類には、各種野菜および果実の切断物、破砕物、磨砕物、搾汁、酵素処理物、それらの希釈物および濃縮物が含まれる。野菜類には、例えばカボチャ、ニンジン、トマト、ピーマン、セロリ、ホウレンソウ、有色サツマイモ、コーン、ビート、ケール、パセリ、キャベツ、ブロッコリーなどが含まれる。果実類には、例えばリンゴ、モモ、バナナ、イチゴ、ブドウ、スイカ、オレンジ、ミカンなどが含まれる。

[0074] 野菜または果実の切断物、破砕物または磨砕物は、例えば上記野菜類または果実類を洗浄後、必要に応じて熱湯に入れるなどのブランチング処理した後、クラッシャー、ミキサー、フードプロセッサー、パルパーフィッシャー、マイコロイダー(Mycolloider™, 特殊機化工業社製)などを用いて切断、破砕、磨砕することによって得ることができる。搾汁は、例えばフィルタープレス、ジューサーミキサーなどを用いて調製することができる。また上記磨砕物を濾布などを用いて濾過することによっても搾汁を調製することができる。酵素処理物は、上記切断物、破砕物、磨砕物、搾汁などにセルラーゼ、ペクチナーゼ、プロトペクチン分解酵素などを作用させることによって調製できる。希釈物には水で1～50倍に希釈したものが含まれる。濃縮物には、例えば凍結濃縮、減圧濃縮などの手段によって1～100倍に濃縮したものが含まれる。

[0075] 発酵用原料物質の他の具体例である豆乳は、常法に従い、大豆原料から調製することができる。該豆乳には、例えば、脱皮大豆を水に浸漬後、コロイドミルなどの適当な粉砕機を用いて湿式粉砕処理後、常法に従いホモジナイズ処理した均質化液、水溶性大豆蛋白質を水中に溶解した溶解液なども包含される。

[0076] 乳酸菌を利用した発酵は、予めスターターを用意し、これを前記各種の発酵用原料物質に接種して発酵させる方法が好ましい。ここでスターターとしては、例えば代表的には予め90～121℃、5～20分間通常の殺菌処理を行った発酵用原料物質を

添加した10%脱脂粉乳培地などに、ONRIC乳酸菌を接種して培養したものを挙げることができる。このようにして得られるスターターは、通常、ONRIC乳酸菌を10^7～10^9cfu／g培養物程度含んでいる。

[0077] スターターに用いる発酵用原料物質には、必要に応じてONRIC乳酸菌の良好な生育のための発酵促進物質、例えばグルコース、澱粉、蔗糖、乳糖、デキストリン、ソルビトール、フラクトースなどの炭素源、ペプトンなどの窒素源、ビタミン類、ミネラル類などを加えることができる。

[0078] 培養条件は、一般に、発酵温度20～45℃程度、好ましくは25～37℃程度、発酵時間10～30時間程度から選ばれる。

[0079] また、本発明茶飲料の他方の必須成分である茶抽出液については、前記本発明茶－発酵飲料において茶発酵液に添加される茶抽出液についてと同様である。

[0080] ONRIC乳酸菌と茶抽出液との配合割合は、例えば、両者を混合して得られる本発明茶飲料が、該乳酸菌を10^4cfu／mL～10^8cfu／mL、好ましくは10^5cfu／mL～10^7cfu／mL含み且つ該茶飲料の茶由来のブリックス度が0．10～0．50、好ましくは0．18～0．30の範囲となるように適宜決定することができるが、これに限定されない。

[0081] かくして、本発明の茶飲料を得ることができる。本発明の茶飲料は、最終的に適当な容器に無菌的に充填して飲料製品とすることができる。この製品は、お茶本来の風味を有しているのに加えて、ONRIC乳酸菌に由来する粘膜免疫賦活作用またはIgA産生亢進作用を有している。

[0082] 本発明茶飲料の摂取量は、これを摂取する生体の年齢、性別、体重、疾患の程度などに応じて適宜決定され、特に限定されるものではないが、一般には、該飲料中に含まれるONRIC乳酸菌数に換算して、1日ヒト1人あたり乳酸菌数が約10^6～10^{10}cfuとなる範囲から選ばれるのがよい。従って、本発明茶飲料の摂取量はまた、該飲料中の菌数に応じて適宜選択され得るが、一般には約50～1000mLの範囲から選択されるのが好ましい。

[0083] 本発明はまた、粘膜免疫賦活作用またはIgA産生亢進作用を有する茶飲料の製造のための、ラクトバチルス ONRIC b0239 (FERM BP-10064)およびラクトバチルス

ONRIC b0240 (FERM BP-10065)からなる群から選択される少なくとも1種の乳酸菌の使用をも提供する。

発明の効果

[0084] 本発明によれば、お茶本来の風味などに悪影響を与えず、優れたIgA産生亢進作用または粘膜免疫賦活作用を有する茶－発酵飲料または茶飲料が提供される。これらの摂取によれば、その優れたIgA産生亢進作用または粘膜免疫賦活作用に基づいて、生体防御作用を強化できると考えられる。

[0085] 本発明の茶－発酵飲料または茶飲料は、原料として用いる茶葉の種類や、茶発酵液、茶抽出液などの配合を変化させることによって、例えば、軽い香りと爽やかな酸味、軽い香りとキレのある味、濃褐色を呈しマイルドな味などを付与し得、お茶本来の飽きのこない味、香りなどを有し得る。

図面の簡単な説明

[0086] [図1]ラクトバチルス ONRIC b0240の投与が、パイエル板細胞からのIgA産生に及ぼす結果を示すグラフである。

[図2]ラクトバチルス ONRIC b0240の投与がIgG産生に及ぼす影響を明らかにするグラフである。

[図3]ラクトバチルス ONRIC b0240死菌(2×10^9cfu相当)摂取21日後のヒト唾液中総S－IgA量の変化を示すグラフである。

発明を実施するための最良の形態

[0087] 以下、本発明を更に詳しく説明するため、本発明の茶－発酵飲料および茶飲料の製造方法を実施例としてあげ、次いで本発明の茶－発酵飲料および茶飲料に利用する特定の乳酸菌につき行った試験例をあげるが、本発明は、これらの例に限定されるものではない。

[0088] 各例中、%は特記しない限り重量%を示す。

実施例 1

[0089] 茶－発酵飲料の製造方法1

(1)凍結保存菌体の製造

前前培養

121℃で15分間オートクレーブ滅菌したMRS液体培地(Difco製)10mLに、－80℃で凍結保存したラクトバチルス ONRIC b0240 (FERM BP-10065)を解凍して100μL加え、このものを33℃で16時間静置培養することにより、本菌株の前前培養液(菌数:約10^9cfu／mL)を調製した。

[0090] 前培養

121℃で15分間オートクレーブ滅菌したMRS液体培地(Difco製)10mLに、前記で調製した前前培養液100μLを接種し、33℃にて16時間静置培養することにより、本菌株の前培養液(菌数:約10^9cfu／mL)を調製した。

[0091] 本培養

121℃で15分間オートクレーブ滅菌したMRS液体培地(Difco製)1Lに、前記で調製した前培養液10mLを接種し、33℃にて16時間静置培養することにより、本菌株の本培養液(菌数:約10^9cfu／mL)を調製した。

[0092] 集菌・洗浄

本培養液1Lを11000回転／分にて10分間遠心分離して菌体を回収し、121℃で15分間オートクレーブ滅菌したリン酸緩衝生理食塩水で洗浄した。さらに遠心分離にて菌体を回収し、再度滅菌済みリン酸緩衝生理食塩水で洗浄後、遠心分離にて菌体を回収した。緑茶エキスパウダー(三栄源社)2gを脱イオン水1000gで溶解後、121℃で15分間オートクレーブ滅菌した液に上記で回収した菌体を懸濁させ、懸濁液を-80℃で保存した。かくして得られた凍結保存菌の菌数は、1．6×10^9cfu／mLであった。

[0093] (2)茶－発酵飲料の製造

緑茶エキスパウダー(三栄源社)3．4gを脱イオン水2000g中に添加後、60℃に加温し、同温度で10分間攪拌してエキスを溶解させた。得られた溶液を室温まで冷却し、滅菌済み試薬瓶に2000mL移した。この溶液の茶由来のブリックス度は0．32であった。

[0094] 凍結保存したラクトバチルス ONRIC b0240 (FERM BP-10065)1．6×10^9cfu／mLの200μLを前記試薬瓶中に接種し、菌接種後の試薬瓶内容物を33℃で16時間

保温して静置培養することにより、緑茶発酵液を調製した。このものの乳酸菌量は10^7cfu／mLであった。

[0095] 一方、ウーロン茶（三井農林社）20gに、93℃の脱イオン水1000mLを加え、6分間隔でスタート時、中間および終了時の3回攪拌した。得られた茶葉を含む液を、ステンレスフィルターで濾過後、濾液を氷浴上で30℃以下に冷却した。冷却後の濾液を脱イオン水で2000mLに希釈した。得られた茶抽出液の茶由来のブリックス度は0．24であった。

[0096] このウーロン茶（茶抽出液）1000mLに前記で調製した緑茶醗酵液1000mLを混合し、混合物に更にビタミンC500mg／Lを添加した。得られた混合液を加熱殺菌し、適当な容器に充填して、本発明の茶－発酵飲料を調製した。

[0097] 得られた本発明の茶－発酵飲料は、軽い香りと爽やかな酸味を有するものであった。そのpHは5．2であり、含まれる本発明の乳酸菌数は$5．2\times10^6$cfu／mLであった。

[0098] 上記で得られた本発明の茶－発酵飲料は、後述する試験例に示すような試験において、優れたIgA産生促進活性及び粘膜免疫賦活活性を有すると認められた。

実施例 2

[0099] 茶－発酵飲料の製造方法2

（1）凍結乾燥菌体の製造

前前培養

121℃で15分間オートクレーブ滅菌したMRS液体培地（Difco製）10mLに、－80℃で凍結保存したラクトバチルス ONRIC b0240 (FERM BP-10065)を解凍して100μL加え、このものを33℃で16時間静置培養することにより、本菌株の前前培養液（菌数：約10^9cfu／mL）を調製した。

[0100] 前培養

121℃で15分間オートクレーブ滅菌したMRS液体培地（Difco製）10mLに、前記で調製した前前培養液100μLを接種し、33℃にて16時間静置培養することにより、本菌株の前培養液（菌数：約10^9cfu／mL）を調製した。

[0101] 本培養

121℃で15分間オートクレーブ滅菌したMRS液体培地(Difco製)1Lに、前記で調製した前培養液10mLを接種し、33℃にて16時間静置培養することにより、本菌株の本培養液(菌数:約10^9cfu／mL)を調製した。

[0102] 集菌・洗浄

本培養液1Lを11000回転／分にて10分間遠心分離して菌体を回収し、121℃で15分間オートクレーブ滅菌したリン酸緩衝生理食塩水で洗浄した。さらに遠心分離にて菌体を回収し、再度滅菌済みリン酸緩衝生理食塩水で洗浄後、遠心分離にて菌体を回収した。緑茶エキスパウダー(三栄源社)20gを脱イオン水1000gで溶解後、121℃で15分間オートクレーブ滅菌した液に上記で回収した菌体を懸濁させ、懸濁液を凍結乾燥した。かくして得られた凍結乾燥菌体粉末中の菌数は、3．7×10^9cfu／gであった。

[0103] (2)茶－発酵飲料の製造

緑茶エキスパウダー(三栄源社)4gを脱イオン水2000g中に添加後、60℃に加温し、同温度で10分間攪拌してエキスを溶解させた。得られた溶液を室温まで冷却し、滅菌済み試薬瓶に2000mL移した。この液の茶由来のブリックス度は0．36であった。

[0104] 凍結乾燥したラクトバチルス ONRIC b0240 (FERM BP-10065)3．7×10^9cfu／gの25mgを前記試薬瓶中に接種し、試薬瓶内容物を33℃で16時間保温して、菌を静置培養した。(想定初期菌数5×10^4cfu／mL)。

[0105] かくして得られた培養液1000mLに、ビタミンC500mgを添加した後、得られた混合液を加熱殺菌し、適当な容器に充填して、本発明の茶－発酵飲料を調製した。

[0106] (3)茶－発酵飲料の品質評価

得られた茶－発酵飲料は、以下の物性を有するものであった。

＜凍結乾燥菌を用いて得られた本発明の発酵茶＞

pH:4．75

菌数:4．1×10^6cfu／mL(乳等省令(食品衛生法)乳酸菌数の測定法による)

濁度:0．038(分光光度計U－3000、日立)

乳酸含量:14．8mg／100mL(高速液体クロマトグラフィ、日本分光)

酢酸含量:0．6mg／100mL(高速液体クロマトグラフィ、日本分光)

クエン酸含量:1．2mg／100mL(高速液体クロマトグラフィ、日本分光)

茶由来のブリックス度:0．24(デジタル示差濃度計DD－5(株式会社アタゴ製)

官能評価:茶の風味があり、酸味が感じられる(供試茶を4℃で試飲させて評価)

上記で得られた本発明茶－発酵飲料は、後述する試験例に示すような試験において、優れたIgA産生促進活性及び粘膜免疫賦活活性を有すると認められた。

実施例 3

[0107] 茶飲料の製造方法1

ウーロン茶(三井農林社)40gに、93℃の脱イオン水1000mLを加え、6分間でスタート時、中間および終了時の3回攪拌した。得られた茶葉を含む液を、ステンレスフィルターで濾過後、濾液を氷浴上で30℃以下に冷却した。得られた茶抽出液の茶由来のブリックス度は0．96であった。

[0108] 一方、予め、人参果汁15%を含む水溶液1000mLに、ラクトバチルス ONRIC b0240 (FERM P-10065)の凍結保存菌(実施例1－(1)で調製したもの)1mLを接種し、33℃で24時間培養後、培養液を遠心分離して60℃、10分間以上で殺菌した後、濃縮菌液(菌体含量:約10^{10}cfu／mL)を調製した。

[0109] 前記ウーロン茶濾液(茶抽出液)1000mLに、上記ラクトバチルス ONRIC b0240の濃縮菌液4mLを添加し、得られた混合液中にビタミンC500mg／Lを添加して、更に水で全量を4000mLに希釈し、得られた希釈液を適当な容器に充填して、本発明茶飲料製品を調製した。

[0110] かくして得られた本発明茶飲料は、軽い香りとキレのある味であった。そのpHは5．5であり、菌数は2．6×10^{7}cfu／mLであった。

[0111] 上記で得られた本発明茶飲料は、後述する試験例に示すような試験において、優れたIgA産生促進活性及び粘膜免疫賦活活性を有すると認められた。

実施例 4

[0112] 茶飲料の製造方法2

実施例3において、原料として利用したウーロン茶に代えてプーアル茶(三井農林社)(得られた茶抽出液の茶由来のブリックス度は0．64である)を用い、ラクトバチル

ス ONRIC b0240 (FERM P-10065)に代えてラクトバチルス ONRIC b0239 (FERM P-10064)(実施例1－(1)と同様にして調製した凍結保存菌体)を用い、同様にして、本発明茶飲料製品を調製した。

[0113] 得られた本発明の茶飲料は、濃褐色を呈しており、マイルドな味で、独特の香りがあった。そのpHは5．5であり、菌数は2．6×10^7cfu／mLであった。

[0114] 上記で得られた本発明茶飲料は、後述する試験例に示すような試験において、優れたIgA産生促進活性及び粘膜免疫賦活活性を有すると認められた。

実施例 5

[0115] 茶－発酵飲料の製造方法3

実施例2において、ラクトバチルス ONRIC b0240 (FERM P-10065)に代えてラクトバチルス ONRIC b0239 (FERM P-10064)を用いて、同様にして、本発明茶－発酵飲料を調製した。

[0116] 得られた本発明茶－発酵飲料は、茶の風味があり、酸味があった。そのpHは5．0であり、菌数は4．0×10^6cfu／mLであった。

[0117] 上記で得られた本発明茶－発酵飲料は、後述する試験例に示すような試験において、優れたIgA産生促進活性及び粘膜免疫賦活活性を有すると認められた。

実施例 6

[0118] 茶－発酵飲料の製造方法4

実施例1において、原料として利用した緑茶エキスパウダーに代えて紅茶抽出エキスパウダーを用い、ウーロン茶に代えて紅茶を用い、ラクトバチルス ONRIC b0240 (FERM P-10065) に代えてラクトバチルス ONRIC b0239 (FERM P-10064)(凍結保存菌体)を用いて、同様にして、本発明の茶－発酵飲料を調製した。

[0119] 得られた本発明の茶－発酵飲料は、紅茶の味を保持したものであった。そのpHは5．8であり、菌数は1．2×10^7cfu／mLであった。

[0120] 上記で得られた本発明の茶－発酵飲料は、後述する試験例に示すような試験において、優れたIgA産生促進活性及び粘膜免疫賦活活性を有すると認められた。

[0121] 試験例1

この例は、ONRIC乳酸菌のIgA産生誘導能を、YasuiらおよびIkenagaらに記載の

方法〔Yasui, H., et al., Microbial Ecology in Health and Disease, 5, 155 (1992); Ikenaga, T., et al., Milk Science, 51, 27 (2002)〕に従ってパイエル板細胞培養系を用いてin vitroで試験した例であり、次の通り実施された。

[0122] (1)供試動物

近交系雌性マウスSPF/VAF BALB/c AnNCrjを使用した。

[0123] 試験マウスを入荷後、1週間検疫した。検疫期間中はMF固形飼料(オリエンタル酵母社製)および水道水を自由摂取させた。

[0124] (2)パイエル板細胞培養法

検疫終了後、各群の体重が均等になるように80匹のマウスを10匹ずつ群分けした。群分け後、毎日10匹のマウスを屠殺し、小腸を取り出し、小腸からパイエル板を切り出し、MEM培地[イーグルMEM (NISSUI社製)、2mM グルタミン (GIBCO社製)、1mM ピルビン酸ナトリウム (GIBCO社製)、MEM非必須アミノ酸 (GIBCO社製)]を添加した遠沈管中で氷冷した。メッシュを用いて単一細胞懸濁液を調製し、5mLのMEM培地でよく洗い込んだ。細胞懸濁液を濾過し、4℃下、1000回転／分、10分間遠心処理を行った。遠心後、培養上清を吸引除去し、沈殿を5mLのMEM培地に懸濁させた。同様の操作を2回繰り返した後、沈殿を10mLの5%FBS(GIBCO社製)含有MEM培地に懸濁させ、パイエル板細胞の生細胞数を計数し、細胞浮遊液を96ウエル細胞培養用プレートに播いて細胞培養用プレートを調製した。

[0125] (3)供試菌体の調製

供試菌体として、ラクトバチルス ONRIC b0239 (FERM BP-10064)およびラクトバチルス ONRIC b0240 (FERM BP-10065)を利用した。各菌は、それぞれその培養に適した培地にて定常期まで培養後、遠心して菌体を集菌した(7000g×10分間、4℃)。PBS(−)にて3回洗浄後、菌体を5mLの生理食塩水に懸濁させた。菌数を把握するため660nmにて濁度を測定し、その後、オートクレーブにて100℃で30分間加熱滅菌処理した。660nmにおける濁度が1.0のとき菌数を2.0×10^9／mLとした。

[0126] (4)培養上清中のIgA濃度の測定

上記(2)で調製したパイエル板細胞を5%FBS含有MEM培地に懸濁させて、2.5×10^6細胞／mLに調整し、その200μLを96ウエル細胞培養用プレートに入れた

。このプレートの各ウエルに2．0×10^9／mLの供試菌体懸濁液（前記（3）で調製したもの）を20μL添加し、37℃、5%CO_2下で7日間培養した。

[0127] 上記菌体20μLに代えて、50μg／mLのLPS(リポポリサッカライド)を20μL添加したものを陽性対照とした。

[0128] 次いで、得られた各培養物上清の総IgA濃度を市販キットを用いたELISA法により測定した。

[0129] (5)ONRIC乳酸菌のIgA産生促進活性

前記（4）に従って測定されたONRIC乳酸菌における総IgA量を、対照としてのMEM培地にPBS（－） 10μLを添加して（菌体無添加）同様にして7日間培養して得た培養物上清の同測定値を基準（1．0）として、その相対比(Stimulation Index; S.I.)にて、下記表1に示す。

[0130] また、下記表1～表4には、既知の各種乳酸菌などについて行った同一試験の結果を併記する。また陽性対照（LPS 50μg／mL）における試験結果を「陽性対照」として併記する。表中、Strain No.に示される微生物保存機関の略号と名称は、それぞれ以下の通りである。

[0131] ATCC: アメリカンタイプカルチャーコレクション (American Type Culture Collection; Manassas, VA, U.S.A.)

JCM: 理化学研究所微生物系統保存施設 (Japan Collection of Microorganism, The Institute of Physical and Chemical Research, RIKEN)

NRIC: 東京農業大学応用生物化学部菌株保存室 (NODAI Culture Collection Center, Tokyo University of Agriculture; Setagaya-ku, Tokyo, Japan)

[0132] [表1]

Strain No.		Genus	Species	Subsp.	IgA S.I.
		対照 (PBS)			1
		陽性対照 (LPS)			13.1
ONRIC	b0239	*Lactobacillus*	*plantarum*		5.61
ONRIC	b0240	*Lactobacillus*	*plantarum*		6.31
ATCC	43121	*Lactobacillus*	*acidophilus*		1.10
JCM	1059	*Lactobacillus*	*brevis*		1.20
JCM	1115	*Lactobacillus*	*buchneri*		1.17
JCM	1134	*Lactobacillus*	*casei*	*casei*	1.03
JCM	1096	*Lactobacillus*	*curvatus*		1.63
JCM	1002	*Lactobacillus*	*delbrueckii*	*bulgaricus*	1.23
JCM	1012	*Lactobacillus*	*delbrueckii*	*delbrueckii*	1.41
JCM	1248	*Lactobacillus*	*delbrueckii*	*lactis*	1.31
JCM	1173	*Lactobacillus*	*fermentum*		1.08
JCM	1131	*Lactobacillus*	*gasseri*		1.15
JCM	1155	*Lactobacillus*	*hilgardii*		1.11
JCM	2012	*Lactobacillus*	*johnsonii*		1.11
JCM	8572	*Lactobacillus*	*kefirgranum*		1.08
JCM	5818	*Lactobacillus*	*kefiri*		1.21
JCM	8130	*Lactobacillus*	*paracasei*	*paracasei*	1.11
JCM	1171	*Lactobacillus*	*paracasei*	*tolerans*	1.11
JCM	1149	*Lactobacillus*	*plantarum*		1.66
JCM	1551	*Lactobacillus*	*plantarum*		1.14
JCM	8341	*Lactobacillus*	*plantarum*		1.18
JCM	1112	*Lactobacillus*	*reuteri*		1.15
ATCC	7469	*Lactobacillus*	*rhamnosus*		1.05
JCM	1157	*Lactobacillus*	*sakei*	*sakei*	1.52
JCM	1150	*Lactobacillus*	*salivarius*	*salicinius*	1.06
JCM	1231	*Lactobacillus*	*salivarius*	*salivarius*	1.14
JCM	9504	*Lactobacillus*	*suebicus*		1.28
JCM	5885	*Pediococcus*	*acidilactici*	(*pentosaceus*)	1.51
JCM	5890	*Pediococcus*	*pentosaceus*		1.44
JCM	6124	*Leuconostoc*	*mesenteroides*	*mesenteroides*	1
NRIC	0103	*Enterococcus*	*faecalis*		1.06
NRIC	0110	*Enterococcus*	*faecalis*		1.08
NRIC	0134	*Lactobacillus*	*brevis*		1.07
NRIC	0137	*Lactobacillus*	*brevis*		1.13
NRIC	1713	*Lactobacillus*	*brevis*		1.08
NRIC	1950	*Lactobacillus*	*brevis*		1.12
NRIC	1964	*Lactobacillus*	*brevis*		1.07
NRIC	1965	*Lactobacillus*	*brevis*		1.07

[0133] [表2]

Strain No.	Genus	Species	Subsp.	IgA S.I.
NRIC1042	*Lactobacillus*	*casei*	*casei*	1.00
NRIC1597	*Lactobacillus*	*casei*	*casei*	0.96
NRIC1917	*Lactobacillus*	*casei*	*casei*	1.01
NRIC1941	*Lactobacillus*	*casei*	*casei*	1.02
NRIC1962	*Lactobacillus*	*casei*	*casei*	1.00
NRIC1963	*Lactobacillus*	*casei*	*casei*	1.05
NRIC1968	*Lactobacillus*	*casei*	*casei*	1.07
NRIC1975	*Lactobacillus*	*curvatus*		1.02
NRIC1976	*Lactobacillus*	*curvatus*		1.14
NRIC1977	*Lactobacillus*	*curvatus*		1.04
NRIC1978	*Lactobacillus*	*curvatus*		1.11
NRIC1979	*Lactobacillus*	*curvatus*		0.99
NRIC0191	*Lactobacillus*	*delbrueckii*	*bulgaricus*	1.07
NRIC1682	*Lactobacillus*	*delbrueckii*	*lactis*	1.12
NRIC0129	*Lactobacillus*	*fermentum*		1.00
NRIC0131	*Lactobacillus*	*fermentum*		1.19
NRIC0132	*Lactobacillus*	*fermentum*		1.03
NRIC0135	*Lactobacillus*	*fermentum*		1.02
NRIC0139	*Lactobacillus*	*fermentum*		1.14
NRIC0141	*Lactobacillus*	*fermentum*		1.08
NRIC0142	*Lactobacillus*	*fermentum*		0.94
NRIC0143	*Lactobacillus*	*fermentum*		1.04
NRIC0144	*Lactobacillus*	*fermentum*		0.97
NRIC0145	*Lactobacillus*	*fermentum*		1.09
NRIC0146	*Lactobacillus*	*fermentum*		1.05
NRIC0147	*Lactobacillus*	*fermentum*		1.05
NRIC1949	*Lactobacillus*	*fermentum*		1.09
NRIC1952	*Lactobacillus*	*fermentum*		1.06
NRIC1955	*Lactobacillus*	*fermentum*		1.12
NRIC1966	*Lactobacillus*	*hilgardii*		0.94
NRIC1967	*Lactobacillus*	*hilgardii*		1.06
NRIC1936	*Lactobacillus*	*paracasei*	*paracasei*	0.96
NRIC1937	*Lactobacillus*	*paracasei*	*paracasei*	0.94
NRIC1942	*Lactobacillus*	*paracasei*	*paracasei*	0.93
NRIC1944	*Lactobacillus*	*paracasei*	*paracasei*	1.00
NRIC1945	*Lactobacillus*	*paracasei*	*paracasei*	0.98
NRIC1946	*Lactobacillus*	*paracasei*	*paracasei*	1.01
NRIC1934	*Lactobacillus*	*paracasei*	*tolerans*	1.09
NRIC1935	*Lactobacillus*	*paracasei*	*tolerans*	1.03
NRIC1938	*Lactobacillus*	*paracasei*	*tolerans*	1.03

[0134] [表3]

Strain No.	Genus	Species	Subsp.	IgA S.I.
NRIC1939	*Lactobacillus*	*paracasei*	*tolerans*	1.01
NRIC1940	*Lactobacillus*	*paracasei*	*tolerans*	1.01
NRIC1943	*Lactobacillus*	*paracasei*	*tolerans*	0.99
NRIC1947	*Lactobacillus*	*paracasei*	*tolerans*	0.98
NRIC0391	*Lactobacillus*	*pentosus*		1.00
NRIC0392	*Lactobacillus*	*pentosus*		1.04
NRIC0393	*Lactobacillus*	*pentosus*		1.19
NRIC0394	*Lactobacillus*	*pentosus*		1.15
NRIC1919	*Lactobacillus*	*plantarum*		1.32
NRIC1920	*Lactobacillus*	*plantarum*		1.08
NRIC1921	*Lactobacillus*	*plantarum*		1.14
NRIC1922	*Lactobacillus*	*plantarum*		1.37
NRIC1923	*Lactobacillus*	*plantarum*		0.96
NRIC1957	*Lactobacillus*	*plantarum*		1.01
NRIC1958	*Lactobacillus*	*plantarum*		1.31
NRIC1715	*Lactobacillus*	*reuteri*		0.95
NRIC1974	*Lactobacillus*	*reuteri*		1.16
NRIC1980	*Lactobacillus*	*reuteri*		1.31
NRIC1599	*Lactobacillus*	*sakei*		0.97
NRIC1600	*Lactobacillus*	*sakei*		1.52
NRIC1601	*Lactobacillus*	*sakei*		1.07
NRIC1602	*Lactobacillus*	*sakei*		1.37
NRIC1603	*Lactobacillus*	*sakei*		1.03
NRIC1575	*Leuconostoc*	*lactis*		0.85
NRIC1576	*Leuconostoc*	*lactis*		0.92
NRIC1578	*Leuconostoc*	*lactis*		1.00
NRIC1580	*Leuconostoc*	*lactis*		1.03
NRIC1582	*Leuconostoc*	*lactis*		0.93
NRIC1750	*Leuconostoc*	*lactis*		1.03
NRIC1087	*Leuconostoc*	*mesenteroides*	*mesenteroides*	1.33
NRIC1507	*Leuconostoc*	*mesenteroides*	*mesenteroides*	1.02
NRIC1541	*Leuconostoc*	*mesenteroides*	*mesenteroides*	0.90
NRIC0124	*Pediococcus*	*acidilactici*		0.93
NRIC0122	*Pediococcus*	*pentosaceus*		1.03
NRIC0123	*Pediococcus*	*pentosaceus*		0.96
NRIC1913	*Pediococcus*	*pentosaceus*		1.62
NRIC1914	*Pediococcus*	*pentosaceus*		1.05
NRIC1915	*Pediococcus*	*pentosaceus*		1.28
NRIC0001	*Saccharomyces*	*cerevisiae*		1.04
NRIC0002	*Saccharomyces*	*cerevisiae*		1.02
NRIC0004	*Saccharomyces*	*Cerevisiae*		1.12

[0135] [表4]

Strain No.	Genus	Species	Subsp.	IgA S.I.
NRIC0005	*Saccharomyces*	*cerevisiae*		1.00
NRIC0006	*Saccharomyces*	*cerevisiae*		1.01
NRIC0007	*Saccharomyces*	*cerevisiae*		0.98
NRIC0008	*Saccharomyces*	*cerevisiae*		0.97
NRIC0009	*Saccharomyces*	*cerevisiae*		0.98
NRIC0011	*Saccharomyces*	*cerevisiae*		1.03
NRIC0013	*Saccharomyces*	*cerevisiae*		0.95
NRIC0014	*Saccharomyces*	*cerevisiae*		0.94
NRIC0015	*Saccharomyces*	*cerevisiae*		1.04
NRIC0016	*Saccharomyces*	*cerevisiae*		0.88
NRIC0059	*Saccharomyces*	*cerevisiae*		1.12
NRIC0060	*Saccharomyces*	*cerevisiae*		1.11
NRIC1412	*Saccharomyces*	*cerevisiae*		1.00
NRIC1414	*Saccharomyces*	*cerevisiae*		1.03
NRIC1415	*Saccharomyces*	*cerevisiae*		0.85
NRIC1417	*Saccharomyces*	*cerevisiae*		0.97
NRIC1461	*Saccharomyces*	*cerevisiae*		0.92
NRIC1465	*Saccharomyces*	*cerevisiae*		1.00
NRIC1466	*Saccharomyces*	*cerevisiae*		1.07
NRIC1624	*Saccharomyces*	*cerevisiae*		0.91
NRIC1478	*Saccharomyces*	*cerevisiae*		0.91
NRIC1482	*Saccharomyces*	*cerevisiae*		0.94
NRIC1483	*Saccharomyces*	*cerevisiae*		1.24
NRIC1484	*Saccharomyces*	*cerevisiae*		0.87
NRIC1485	*Saccharomyces*	*cerevisiae*		0.95
NRIC1486	*Saccharomyces*	*cerevisiae*		1.04
NRIC1487	*Saccharomyces*	*cerevisiae*		0.91
NRIC1488	*Saccharomyces*	*cerevisiae*		0.91
NRIC1489	*Saccharomyces*	*cerevisiae*		0.84
NRIC1490	*Saccharomyces*	*cerevisiae*		0.88
NRIC1811	*Saccharomyces*	*cerevisiae*		1.03

[0136] 表1～4に示されるとおり、対照(PBS)のIgA産生を1とした場合、陽性対照の平均S. I. は、13. 1を示し、IgA産生を強く誘導していることが判った。よって本培養系はパイエル板細胞からのIgA産生を評価する上で有用であると判断した。

[0137] 各種乳酸菌によるIgA産生誘導能を見ると、S. I. は、ラクトバチルス ONRIC b0239が5. 61、ラクトバチルス ONRIC b0240が6. 31であり、他の菌株の0. 8～1. 4に比べて突出して高いIgA産生誘導能を有することが判った。

[0138] IgAは病原微生物の粘膜からの侵入阻止、ウイルス・毒素の中和、食物アレルゲンの侵入阻止などの働きをしており、このような機能を有するIgAの産生を高めておくことは生体防御の上で重要である。

[0139] 試験例2

この例は、ラクトバチルス ONRIC b0240 (FERM BP-10065)のIgA産生誘導能をin vivoで試験したものであり、次の通り実施された。

[0140] (1)供試動物およびその飼育

8週齢BALB/c雄性マウス50匹を入荷後、1週間検疫した。検疫期間中および引き続く試験期間中、実験動物にはMF固形飼料(オリエンタル酵母社製)および水道水を自由摂取させた。

[0141] 検疫終了後、各実験動物を生理食塩水投与群(15匹)、ラクトバチルス ONRIC b0240(生菌)投与群(15匹)およびラクトバチルス ONRIC b0240(死菌)投与群(15匹)に群分けた。

[0142] (2)経口投与用ラクトバチルス ONRIC b0240の調製

経口投与用のラクトバチルス ONRIC b0240(生菌)およびラクトバチルス ONRIC b0240(死菌)は、それぞれ以下の方法により調製した。

[0143] 生菌:

ラクトバチルス ONRIC b0240をMRS培地にて定常期まで培養した後、遠心(3500回転/分×10分、4℃)にて集菌した。生理食塩水にて2回遠心洗浄後、菌体を生理食塩水に懸濁して、4×10^{9}cfu/mLに調整した。

[0144] 死菌

上記で得た生菌懸濁液を、オートクレーブ(121℃、15分加熱)処理後、洗浄用ソニケーター(BRANSON 2510)で45分間超音波処理を行った。

[0145] (3)試験方法

上記(2)で調製したラクトバチルス ONRIC b0240(生菌および死菌)のそれぞれを、試験開始より7日間(5匹)、14日間(5匹)または21日間(5匹)に亘って、ラクトバチルス ONRIC b0240(生菌)投与群(5+5+5=15匹)およびラクトバチルス ONRIC b0240(死菌)投与群(5+5+5=15匹)の各群マウスに、毎朝、経口投与(10^{9}CFU

／250μL／匹／日）した。各投与期間終了後に、各群マウスを断頭屠殺してチューブに採血し、4℃下、3000回転／分、10分間遠心分離して血清を調製した。また、以下の方法によりパイエル板細胞を調製した。即ち、各群マウスを屠殺後、小腸を摘出し、眼科用ハサミで小腸からパイエル板を切り出し、不完全培地(incomplete medium; 10mg ゲンタマイシン添加RPMI1640培地)を添加した24ウェルマイクロプレートに入れて氷冷した。メッシュを用いて単一細胞懸濁液を調製し、5mLの不完全培地で良く洗った。得られた細胞浮遊液を濾過し、4℃下、1000回転／分で10分間遠心分離処理した。遠心分離処理後、培養上清を吸引除去し、沈殿を5mLの不完全培地に懸濁させた。上記洗浄、濾過、遠心分離、培養上清の吸引除去操作を、更に1回繰り返した後、得られた沈殿をパイエル板細胞とした。

[0146] また、コントロールとしての生理食塩水投与群（15匹）のマウスは、ラクトバチルス ONRIC b0240（生菌および死菌）を与えることなく飼育し、同様に試験開始より7日間（5匹）、14日間（5匹）および21日間（5匹）後に、血清およびパイエル板細胞を調製した。

[0147] IgA産生試験

調製した各パイエル板細胞（沈殿）を、0．5mLの完全培地(2mM Lーグルタミン、50μMメルカプトエタノール、100U／mLペニシリン、100μg／mLストレプトマイシン、10%FBS添加RPMI1640培地)に懸濁させて、細胞濃度を2×10^6細胞／mLに調整し、生細胞数をカウント後、細胞浮遊液を100μLずつ96ウエル細胞培養用プレートの各ウエルに播種した。

[0148] パイエル板細胞が産生するIgA量は、パイエル板細胞をそのまま培養し、産生されるIgA量を調べる方法と、この培養系に更にパイエル板細胞刺激物質としてラクトバチルス ONRIC b0240（死菌）を添加して培養し、産生されるIgA量を調べる方法との2つの方法により検討した。後者の方法は、実際の生体内におけるパイエル板細胞の環境に近いものと想定される。即ち、この試験でラクトバチルス ONRIC b0240（生菌または死菌）を経口投与する場合は、摂取された乳酸菌は何らかの形でパイエル板細胞に刺激を与えることが予想される。

[0149] パイエル板細胞刺激物質としてのラクトバチルス ONRIC b0240（死菌）は、下記方

法に従って調製した。

[0150] パイエル板細胞刺激用ラクトバチルス ONRIC b0240(死菌)

前記で調製した経口投与用のラクトバチルス ONRIC b0240(生菌)の懸濁液を、更にリン酸バッファーで菌数が10^7cfu／mL(660nmでの濁度0．275)となるように希釈し、得られた菌体懸濁液を、オートクレーブ(121℃、15分加熱)処理後、洗浄用ソニケーター(BRANSON 2510)で45分間超音波処理した。

[0151] パイエル板細胞刺激物質を利用する方法は、パイエル板細胞刺激用ラクトバチルス ONRIC b0240(死菌)10μLを各ウエルに添加し、さらにFCSを含まないRPMI1640を100μL各ウエルに添加し、37℃、5%CO_2下で7日間パイエル板細胞を培養した。パイエル板細胞刺激物質を利用しない方法では、上記ラクトバチルス ONRIC b0240(死菌)の代わりに10μLの生理食塩水を各ウエルに添加して、以下同様の操作によってパイエル板細胞を培養した。

[0152] (4)測定

細胞培養液から遠心分離にて培養上清を回収し、該培養上清中に分泌される総IgA濃度の測定に供するまで－80℃で凍結保存した。

[0153] 上記培養上清中の総IgA濃度の測定および血清中の総IgG濃度の測定は、いずれも市販のキットを用いたELISA法により測定した。

[0154] (5)結果

結果を図1(IgA濃度)および図2(IgG濃度)に示す。

[0155] 図1は、培養上清中のIgA濃度(μg／mL)を示す棒グラフである。図中、白抜き棒は、コントロールとしての生理食塩水投与群(「生理食塩水」と表示)の結果である。網掛け棒は、ラクトバチルス ONRIC b0240 (生菌)投与群(「b0240生菌」と表示)の結果である。黒塗り棒は、ラクトバチルス ONRIC b0240 (死菌)投与群(「b0240死菌」と表示)の結果である。無刺激は、各群マウス由来のパイエル板細胞の培養系にラクトバチルス ONRIC b0240 (死菌)を添加することなく培養した場合を示す。菌体刺激は、各群マウス由来のパイエル板細胞の培養系にラクトバチルス ONRIC b0240 (死菌)を添加して該菌の刺激下に培養した場合を示す。各結果は、各群供試マウス5匹について得られた結果を平均±標準偏差(Mean±SD)で表示する。各結果の上に表示し

たP値は、スチューデンツt-テスト(Student t-test)におけるコントロールに対する危険率を示す。

[0156] 該図に示される結果から、次のことが明らかである。

[0157] (1)7日間投与の場合:

菌体刺激の場合、ラクトバチルス ONRIC b0240(死菌)投与群は、生理食塩水投与のコントロールよりも有意に高値を示した(P=0.010)。

[0158] (2)14日間投与の場合:

無刺激の場合、ラクトバチルス ONRIC b0240(死菌)投与群(無刺激の黒塗り棒参照)は、コントロール(生理食塩水投与後無刺激)よりも、有意に高値を示した(P=0.048)。

[0159] また、菌体刺激を行った場合は、ラクトバチルス ONRIC b0240(死菌)を投与した群およびラクトバチルス ONRIC b0240(生菌)を投与した群のいずれも、コントロール(生理食塩水投与)に比して有意に高値を示した(それぞれp=0.034およびp=0.002)。

[0160] (3)21日間投与の場合:

無刺激の場合、ラクトバチルス ONRIC b0240(死菌)を投与した群は、コントロール群よりも有意に高値を示した(P=0.047)。

[0161] また、菌体刺激を行った場合、ラクトバチルス ONRIC b0240(生菌)投与群およびラクトバチルス ONRIC b0240(死菌)投与群は、コントロール群よりもいずれも有意に高値を示した(それぞれp=0.015およびp=0.005)。

[0162] 図2は、ラクトバチルス ONRIC b0240(死菌)の21日間投与がIgG産生に及ぼす影響を明らかにする棒グラフであり、縦軸は血清IgG濃度(μg/mL)を示す。

[0163] 該図に示される結果から、ラクトバチルス ONRIC b0240(死菌)の投与は、コントロール(生理食塩水投与)に比して有意に高い血清IgG濃度を示すことが判る(p=0.0064)。またラクトバチルス ONRIC b0240(生菌)の投与も、コントロール(生理食塩水投与)に比して、高い血清IgG濃度を示すことが判る。

[0164] 以上の結果より、ラクトバチルス ONRIC b0240は、パイエル板に存在する免疫担当細胞あるいは腸管上皮細胞とその周辺の免疫担当細胞を刺激することで粘膜免疫

応答を誘導し、最終的にパイエル板細胞からの総IgA産生を高めたものと推定される。また、ラクトバチルス ONRIC b0240の投与は、IgAのみならず血清中のIgGも高めることが判った。

[0165] これらのことから、ONRIC乳酸菌の摂取は、粘膜免疫のみならず全身免疫も賦活し、これらの2段構えで生体の免疫応答を賦活し、体の内と外から生体を防御する可能性が示唆される。このような作用が生菌のみならず死菌においても認められることから、ONRIC乳酸菌は、経口ワクチン的なプロバイオティクスの新たな活用法として期待できるものと考えられる。

[0166] 試験例3

この試験はONRIC乳酸菌の摂取がインフルエンザ下気道感染の防御に有効であることを明らかにするものである。

[0167] 本発明者らは、ONRIC乳酸菌のIgAを介した感染防御効果を明らかにするため、インフルエンザウイルス(IFV)を下気道にまで到達させる下気道感染モデルマウスを用いて、ラクトバチルス ONRIC b0240を利用して調製した発酵物の摂取による感染防御効果を、感染後の生存日数を指標として検討した。本試験は以下の通り実施された。

[0168] (1)供試動物

日本チャールス・リバー株式会社より入荷した近交系雌性SPF/VA/VAF マウス(系統名:BALB/c AnNCrj)(5週齢)を4日間、以下の条件で検疫後、体重による群分け(蒸留水群、牛乳群およびラクトバチルス ONRIC b0240含有発酵乳群)を行った。

餌/給餌法:MF固形飼料(オリエンタル酵母株式会社)/自由摂取

水/給水法:水道水/給水瓶による自由摂取

環境:温度:23±2° C、湿度:60±10%

照明時間:明期 7:00～19:00、暗期 19:00～7:00

(2)試験方法

各群マウス(n=45)に、MF固形飼料(オリエンタル酵母社製)と共に、被験物((1)蒸留水、(2)牛乳または(3)ラクトバチルス ONRIC b0240含有発酵乳)を、2週間摂取させた。

[0169] 被験物としての牛乳は、LL牛乳(大阿蘇牛乳:らくのうマザーズ社製)を蒸留水で75%に希釈して利用した。被験物としてのラクトバチルス ONRIC b0240含有発酵乳は、予め10%スキムミルク水溶液に懸濁させ-80℃で凍結保存しておいたラクトバチルス ONRIC b0240 をスターターとして、牛乳1Lに該スターター(生菌数10^8cfu)を加え、33℃で16時間発酵させたものである。該ラクトバチルス ONRIC b0240含有発酵物の菌体含量は5×10^7cfu/mLである。これを蒸留水で75%に希釈して試験に利用した。

[0170] 被験物は給水瓶による自由摂取とし、摂取量は摂取前後の被験物の重量減少量とした。

[0171] 摂取開始2週間後に、各群マウスをケタラール(塩酸ケタミン)により麻酔後、その一方の鼻腔にIFV液50μLを経鼻接種(10、10^2または10^3pfu/50μL PBS/匹、それぞれの濃度におけるn=15)して、IFVをマウスに感染させた。その後、各群実験動物の生死を毎日観察した。被験物は感染後から死亡を確認するまで与え続けた。

[0172] 使用IFV株としては、大塚製薬株式会社微生物研究所に保存されているIFV: A/PR/8/34/H1N1株を用いた。該株を0.1%BSAおよび10mM HEPESを含有するMEM培地に懸濁させ、次いでPBS(+)を用いてウイルス含量が10~10^3pfu/50μLとなるように希釈し、IFVの接種用ウイルス液を調製した。なお、PBS(+)はPBS(-)粉末(コージンバイオ社製)9.55g、$CaCl_2$(無水)100.00mgおよび$MgCl_2$(無水)46.90mgを蒸留水に溶解して1000mLとして調製した。

[0173] (3)結果

各群マウスの生存日数を、IFV経鼻摂取後、毎日朝(8:30~9:00)および夕方(17:30~18:00)の二回、確認した。

[0174] その結果、蒸留水を摂取させたコントロール群および牛乳を摂取させた対照群では、接種ウイルス量を10^2pfu/匹とした場合、いずれも7日目までに全例が死亡した。接種ウイルス量を10^3pfu/匹とした場合、いずれも6日目夕方までに全例が死亡した。これに対して、ラクトバチルス ONRIC b0240含有発酵乳を摂取させた群では、コントロール群に対して、実験動物の生存日数を延長する傾向が認められた。

[0175] また、接種ウイルス量を10pfu／匹とした場合、全ての群において14日目で70%以上が生存しており、ラクトバチルス ONRIC b0240含有発酵乳を摂取させた群では、86．7%が生存しており、コントロール群のそれ(80%)に対して、生存率を延長させる傾向が認められた。

[0176] また、各群マウスの体重を被験物摂取開始から感染日までは2日毎に、感染後は毎朝(8:30～9:00)、電子天秤を用いて測定した。なお、測定は各測定日において生存していたマウスについて行い、得られた値は全測定値の平均値で示した。

[0177] その結果、全ての群において、2日目から若干の体重減少が認められた。体重変化の推移は、各群において同様であり、差は認められなかった。

[0178] (4)考察

本試験の結果および前記試験例1および2に示される試験の結果から、総合的に判断して、ONRIC乳酸菌およびこれを含む発酵物は、IFV感染に対して感染防御効果を奏し得ると考えられる。

[0179] 本発明は、免疫賦活作用およびIgA産生促進作用を有するONRIC乳酸菌を含む茶ー発酵飲料および茶飲料を提供するものであり、これらはその摂取によって病原微生物などの粘膜からの侵入を阻止する生体防御効果を奏することが期待できる。

[0180] 試験例4

この試験はONRIC乳酸菌死菌の摂取がインフルエンザ下気道感染の防御に有効であることを明らかにするものである。

[0181] (1)被験物質

ラクトバチルス ONRIC b0240死菌を被験物質として用いた。

[0182] (2)供試動物

日本エスエルシー株式会社より入荷した雌性BALB/c/Cr Slc(SPF)マウス(5週齢)を7日間、以下の条件で検疫後、群分けを行った。

餌／給餌法:固形飼料 CRF－1(オリエンタル酵母工業株式会社)／自由摂取

水／給水法:123～124℃、100分間高圧蒸気滅菌済み水道水／給水瓶による自由摂取

環境:温度:23±2℃、湿度:55±15%、換気:15回／時間

照明時間：明期8：00～20：00、暗期20：00～8：00。

[0183] (3)ウイルスの調製

超低温冷凍庫に凍結乾燥保存してあるインフルエンザウイルスA型(IFVA：PR8株)を細胞増殖用培地中(10%FBS含有イーグルMEM(Gibco))でMDCK細胞(イヌ腎細胞；RCB0995株)にM.O.I.(Multiplicity of infection)＝0．01で感染させ、37℃、5%CO_2存在下で72時間培養した(1継代)。連続して5代継代したものを大量培養し、蔗糖密度勾配超高速遠心法によりウイルス液を分離・精製し、1mLずつ分注後－80℃超低温冷凍庫にて実験使用時まで保存した。ウイルス液の一部は、10倍段階希釈法にて細胞変性効果を確認し、ウイルス感染力価($TCID_{50}$)を測定した。

[0184] (4)試験方法

(4－1)群分け

群構成は、ウイルス摂取群4群およびウイルス非接種群2群の計6群とした。ウイルス接取群の4群の接取ウイルス濃度は$10^{7.5}TCID_{50}$／mLとした。ウイルス接取が確実に行われたマウスを各群に10匹ずつ割り付けた。なお、群分けの指標には動物番号を用い、被験物質投与開始時に層別無作為に割り付けた。ウイルス非接取群の動物数も10匹とした。

[0185] (4－2)被験物質の投与

検疫・検収の終了後、1週間予備飼育を行った6週齢のBALB/c雌マウスに、被験物質をマウス1匹当たり0．2mLずつ、試験期間を通じて1日1回強制経口投与した。被験物質の濃度は、マウスの体重1kgあたりの被験物質投与量が、500、100または20mg／kgとなるように設定した。なお、陰性対象には生理食塩液を同様に投与した。

[0186] (4－3)IFVAの接種

被験物質または生理食塩液を3週間連続強制経口投与した6週齢のBALB/c雌マウスに、動物番号順に動物体重20g当たり、塩酸ケタミン注射液(50mg／mL；三共株式会社)20倍希釈液0．2mLとドロペリドール注射液(2．5mg／mL；三共株式会社)20倍希釈液0．1mLを後肢筋肉内に注射し、全身麻酔を施した。麻酔下において、上記(3)で調製したIFVA液をマウスの右鼻腔に50μL経鼻的に接種した。なお

、ウイルス非接種群には生理食塩液50μLを同様に注入した。

[0187] (4－4)生存率の測定

IFVA接種後2週間の経過観察を行った。IFVA接種後生存した個体数を群当たりの総数で除した値に100を乗じた値を生存率とした。

[0188] (5)統計処理

実験対照群と各被験物質投与群間の有意差は、クラスカルーウォリスのH検定またはマンーホイットニーのU検定を用いて検定し、危険率5%未満を有意とした。また、観察期間における生存期間に関してはフィッシャー検定による有意差検定を行った。

[0189] (6)結果

結果を表5に示す。

[0190] [表5]

	ラクトバチルス ONRIC b0240 死菌の用量 (mg/kg body weight)	マウス数	生存率	生存期間 (日)
IFVA 接種群	500	10	8/10 (80.0%)	13.1
	100	10	3/10 (30.0%)	9.2
	20	10	0/10 (0.0%)	8.5
	−(生理食塩液)	10	0/10 (0.0%)	8.5
IFVA 非接種群	500	10	10/10 (100.0%)	14.0
	−(生理食塩液)	10	10/10 (100.0%)	14.0

[0191] ウイルス感染動物における生理食塩水投与群の生存率は0%、平均生存日数は8.5日であった。一方、ウイルス感染動物における被験物質投与群の生存率および平均生存日数は、500mg／kg投与群80.0%、13.1日、100mg／kg投与群30.0%、9.2日および20mg／kg投与群0%、8.5日であった。ウイルス感染後の観察期間中の生存期間および観察期間終了時の生存率において、500mg／kg投与群は対照群に対して有意に高い値を示した(それぞれ、p＝0.0002およびp＝0.0007)。また、ウイルス未感染動物における被験物質投与群および同物質非投与群の生存率および平均生存日数に関しては、いずれも100%、14.0日であった。

[0192] 試験例5

この試験は、ONRIC乳酸菌(死菌)の継続摂取が、ヒトの唾液中IgA産生量に及ぼす影響を明らかにするものである。

[0193] 被験者は成人女性20名とし、スクリーニング時の唾液中総S－IgA量を指標に、水摂取群(対照群)、ラクトバチルス ONRIC b0240死菌(2×10^9CFU相当／日)摂取群に各10名を割り付けた。

[0194] 試験期間中は、水またはラクトバチルス ONRIC b0240死菌含有水(菌数2×10^9CFU相当:200ml／日)を摂取させた。被験物質摂取前後に、下記の要領で唾液中総S－IgA量を測定した。

[0195] 唾液採取日の定刻に蒸留水にて洗口し、5分後、サリベットを用いて唾液を1分間採取し、秤量した。唾液分泌量に応じて2～3回この作業を繰り返した。この際、正確な採取時間をタイマーにより計測し、記録した。得られた唾液は遠心分離を行うまで4℃で冷蔵保存した。その日のうちにサンプルを2500rpmにて10分間遠心分離を行い、沈殿物を取り除いた。得られる上清の量が少ない場合はさらに同条件で遠心分離を行った。上清を1．5ml容マイクロチューブに採取し、測定時まで－30℃で凍結保存した。測定には、上清をブロッキング液(魚ゼラチン(SIGMA, St. Louis, MO)1g／50mlを含む、0．05%ポリオキシエチレンソルビタンモノウレート含有PBS(Tween20相当品、ナカライテスク、京都))で2000倍希釈したサンプルを用いた。IgA濃度はELISA法(一次抗体:ウサギ抗ヒトIgA(DAKO、Denmark)、二次抗体:HRP標識ウサギ抗ヒトIgA(DAKO)、標準抗体:精製ヒト分泌型IgA(Cappel, Aurora, OH))にて測定した。なお、総S－IgA量は、一分間あたりの唾液分泌量とS－IgA濃度の積として算出した。

[0196] 試験期間中の唾液中総S－IgA量の増加量を、被験物質摂取21日目における唾液中総S－IgA量から、被験物質摂取前の唾液中総IgA量を引いた差として算出した。得られた結果を図3に示す。試験期間中の唾液中総S－IgA量の増加量について、ラクトバチルス ONRIC b0240死菌(2×10^9cfu相当)摂取群(52．21±24．41μg)は、水摂取群(－21．81±28．33μg)に比べて高い値を示し、その差は統計学的にも有意なものであった(p＝0．0001)。

請求の範囲

[1] ラクトバチルス ONRIC b0239 (FERM BP-10064)およびラクトバチルス ONRIC b0240 (FERM BP-10065)からなる群から選択される少なくとも1種の乳酸菌の茶発酵液を含有することを特徴とする茶－発酵飲料。

[2] 更に茶抽出液を含む請求項1に記載の茶－発酵飲料。

[3] 乳酸菌を、粘膜免疫賦活作用を発揮する有効量含有する請求項1に記載の茶－発酵飲料。

[4] 乳酸菌を、IgA産生亢進作用を発揮する有効量含有する請求項1に記載の茶－発酵飲料。

[5] 乳酸菌を、茶－発酵飲料中に10^4cfu／mL～10^8cfu／mL含有する請求項1に記載の茶－発酵飲料。

[6] 乳酸菌を、茶－発酵飲料中に10^5cfu／mL～10^7cfu／mL含有する請求項1に記載の茶－発酵飲料。

[7] ラクトバチルス ONRIC b0239 (FERM BP-10064)およびラクトバチルス ONRIC b0240 (FERM BP-10065)からなる群から選択される少なくとも1種の乳酸菌および茶抽出液を含有することを特徴とする茶飲料。

[8] 乳酸菌を、粘膜免疫賦活作用を発揮する有効量含有する請求項7に記載の茶飲料。

[9] 乳酸菌を、IgA産生亢進作用を発揮する有効量含有する請求項7に記載の茶飲料。

[10] 乳酸菌を、茶飲料中に10^4cfu／mL～10^8cfu／mL含有する請求項7に記載の茶飲料。

[11] 乳酸菌を、茶飲料中に10^5cfu／mL～10^7cfu／mL含有する請求項7に記載の茶飲料。

[12] 茶含有培地でラクトバチルス ONRIC b0239 (FERM BP-10064)およびラクトバチルス ONRIC b0240 (FERM BP-10065)からなる群から選択される少なくとも1種の乳酸菌を培養する工程を含む、請求項1に記載の茶－発酵飲料の製造方法。

[13] 茶－発酵飲料の乳酸菌含有量を、10^4cfu／mL～10^8cfu／mLに調整する工程を更に含む、請求項12に記載の方法。

[14] 茶含有培地が、任意成分を含んでいてもよい、茶由来のブリックス度0．10～0．50の茶抽出液であり且つ培養が、25℃～50℃の温度で12時間～32時間を要して行われる請求項12に記載の製造方法。

[15] 茶含有培地が、任意成分を含んでいてもよい、茶由来のブリックス度0．18～0．30の茶抽出液であり且つ培養が、30℃～40℃の温度で15時間～20時間を要して行われる請求項12に記載の製造方法。

[16] 請求項2に記載の茶－発酵飲料の製造方法であって、下記の工程：

(1)茶含有培地でラクトバチルス ONRIC b0239 (FERM BP-10064)およびラクトバチルス ONRIC b0240 (FERM BP-10065)からなる群から選択される少なくとも1種の乳酸菌を培 養して茶発酵液を得る工程、

および、

(2)上記(1)の工程によって得られた茶発酵液に茶抽出液を添加する工程、

を含む方法。

[17] (2)の工程において、茶抽出液を、最終的な茶－発酵飲料の茶由来のブリックス度が0．10～0．50となり、且つ乳酸菌含有量が10^4cfu／mL～10^8cfu／mLとなるように茶発酵液に添加する、請求項16に記載の方法。

[18] 請求項7に記載の茶飲料の製法であって、ラクトバチルス ONRIC b0239 (FERM BP-10064)およびラクトバチルス ONRIC b0240 (FERM BP-10065)からなる群から選択される少なくとも1種の乳酸菌と茶抽出液とを混合する工程を含む茶飲料の製造方法。

[19] 茶抽出液を、最終的な茶飲料の茶由来のブリックス度が0．10～0．50となり、且つ乳酸菌含有量が10^4cfu／mL～10^8cfu／mLとなるように乳酸菌と混合する、請求項18に記載の方法。

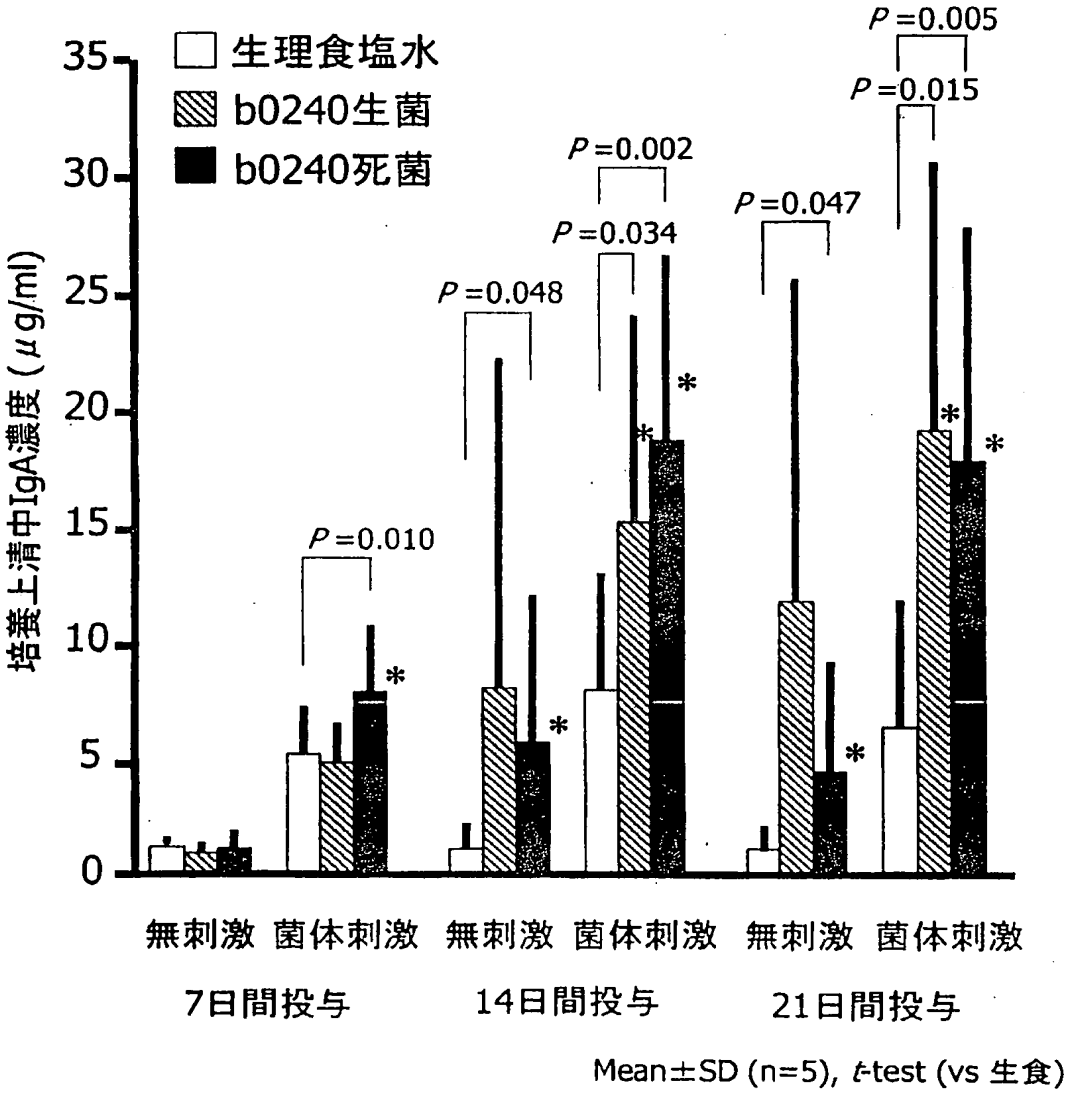
[20] 粘膜免疫賦活作用を有する茶－発酵飲料の製造のための、ラクトバチルス ONRIC b0239 (FERM BP-10064)およびラクトバチルス ONRIC b0240 (FERM BP-10065)からなる群から選択される少なくとも1種の乳酸菌の使用。

[21] IgA産生亢進作用を有する茶－発酵飲料の製造のための、ラクトバチルス ONRIC b0239 (FERM BP-10064)およびラクトバチルス ONRIC b0240 (FERM BP-10065)からなる群から選択される少なくとも1種の乳酸菌の使用。

[22] 粘膜免疫賦活作用を有する茶飲料の製造のための、ラクトバチルス ONRIC b0239 (FERM BP-10064)およびラクトバチルス ONRIC b0240 (FERM BP-10065)からなる群から選択される少なくとも1種の乳酸菌の使用。

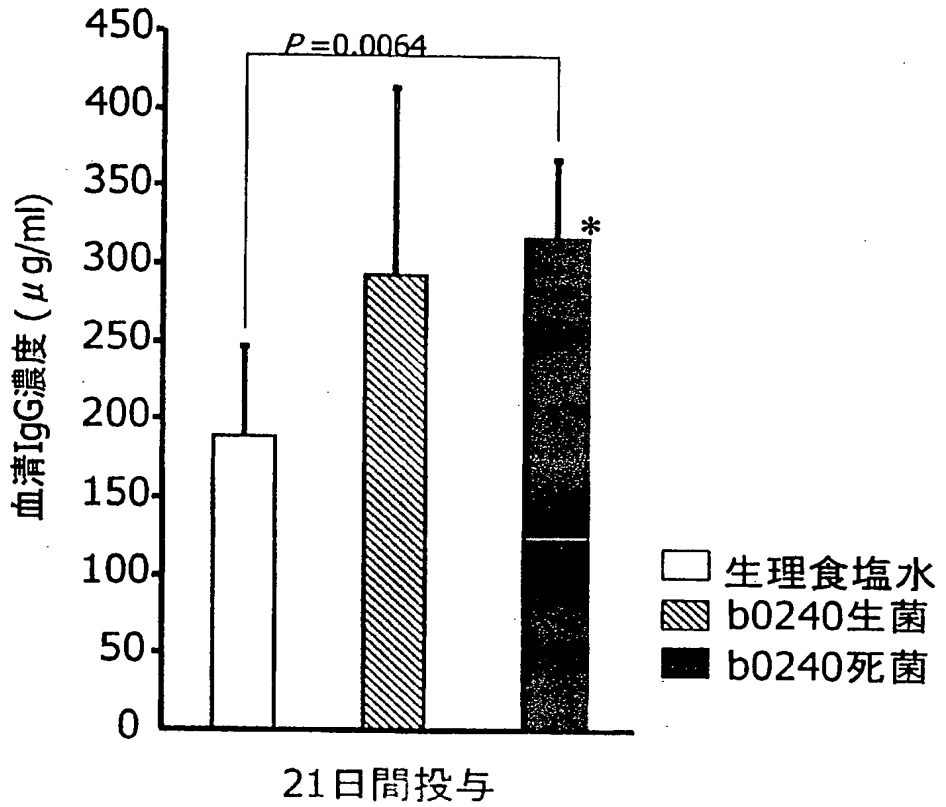
[23] IgA産生亢進作用を有する茶飲料の製造のための、ラクトバチルス ONRIC b0239 (FERM BP-10064)およびラクトバチルス ONRIC b0240 (FERM BP-10065)からなる群から選択される少なくとも1種の乳酸菌の使用。

[図1]



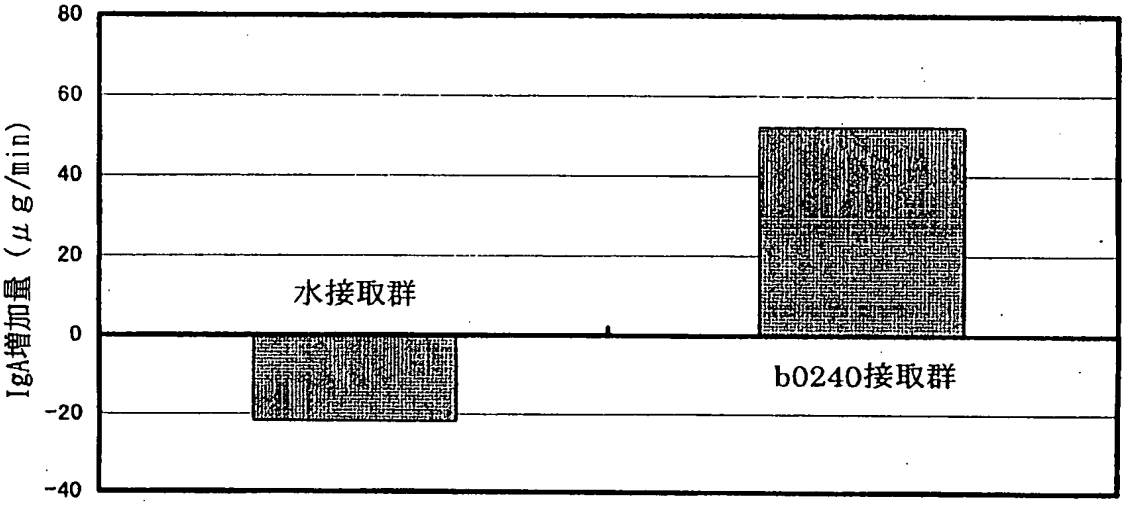
Mean±SD (n=5), *t*-test (vs 生食)

[図2]



Mean±SD (n=5), *t*-test (vs 生食)

[図3]



摂取２１日後におけるヒト唾液中総ＩｇＡ量の変化

INTERNATIONAL SEARCH REPORT

International application No.
PCT/JP2006/303145

A. CLASSIFICATION OF SUBJECT MATTER
A23F3/16(2006.01), *A61K35/74*(2006.01), *A61K36/18*(2006.01), *A61P31/04*(2006.01), *A61P31/12*(2006.01), *A61P37/02*(2006.01)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
A23F3/16(2006.01), *A61K35/74*(2006.01), *A61K36/18*(2006.01), *A61P31/04*(2006.01), *A61P31/12*(2006.01), *A61P37/02*(2006.01)

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
Jitsuyo Shinan Koho 1922-1996 Jitsuyo Shinan Toroku Koho 1996-2006
Kokai Jitsuyo Shinan Koho 1971-2006 Toroku Jitsuyo Shinan Koho 1994-2006

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	JP 2876006 B1 (Kabushiki Kaisha Baiotekku Japan), 31 March, 1999 (31.03.99), Claims 1, 3 to 4; Par. No. [0014] & JP 11-276072 A	1-23
Y	T. IKENAGA et al., Enhancement of host resistance against Salmonella typhimurium in mice fed a diet supplemented with milk fermented with Lactobacillus plantarum., Milk Science(2002), Vol.51, No.1, pages 27 to 32	1-23

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
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"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
09 May, 2006 (09.05.06)	23 May, 2006 (23.05.06)
Name and mailing address of the ISA/ Japanese Patent Office	Authorized officer
Facsimile No.	Telephone No.

Form PCT/ISA/210 (second sheet) (April 2005)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/JP2006/303145

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Y. MAO et al., Intestinal Immune Response to Oral Administration of Lactobacillus reuteri R2LC, Lactobacillus plantarum DSM 9843, Pectin and Oatbase on Methotrexate-induced Enterocolitis in Rats., Microbial Ecology in Health and Disease(1996), Vol.9, No.6, pages 261 to 269	1-23
Y	JP 2002-080364 A (Takeda Food Products, Ltd.), 19 March, 2002 (19.03.02), Full text (Family: none)	1-23
Y	Maria E. Bibas BONET et al., Optimal Effect of Lactobacillus delbrueecki subsp. bulgaricus, Among Other Lactobacilli Species, on the Number of IgA and Mast Cells Associated with the Mucosa in Immunosuppressed Mice., Food and Agricultural Immunology(1999), Vol.11, No.3, pages 259 to 267	1-23
Y	M.V. HERIAS et al., Immunomodulatory effects of Lactobacillus plantarum colonizing the intestine of gnotobiotic rats., Clin.Exp.Immunol.(1999), Vol.116, No.2, pages 283 to 290	1-23
Y	JP 10-167972 A (Takeda Food Products, Ltd.), 23 June, 1998 (23.06.98), Full text (Family: none)	1-23
Y	JP 2001-064174 A (Takeda Food Products, Ltd.), 13 March, 2001 (13.03.01), Full text (Family: none)	1-23

国際調査報告

国際出願番号　ＰＣＴ／ＪＰ２００６／３０３１４５

Ａ．　発明の属する分野の分類（国際特許分類（ＩＰＣ））

Int.Cl.　*A23F3/16 (2006.01), A61K35/74 (2006.01), A61K36/18 (2006.01), A61P31/04 (2006.01), A61P31/12 (2006.01), A61P37/02 (2006.01)*

Ｂ．　調査を行った分野

調査を行った最小限資料（国際特許分類（ＩＰＣ））

Int.Cl.　*A23F3/16 (2006.01), A61K35/74 (2006.01), A61K36/18 (2006.01), A61P31/04 (2006.01), A61P31/12 (2006.01), A61P37/02 (2006.01)*

最小限資料以外の資料で調査を行った分野に含まれるもの

日本国実用新案公報	１９２２－１９９６年
日本国公開実用新案公報	１９７１－２００６年
日本国実用新案登録公報	１９９６－２００６年
日本国登録実用新案公報	１９９４－２００６年

国際調査で使用した電子データベース（データベースの名称、調査に使用した用語）

Ｃ．　関連すると認められる文献

引用文献のカテゴリー＊	引用文献名　及び一部の箇所が関連するときは、その関連する箇所の表示	関連する請求の範囲の番号
Y	JP 2876006 B1 (株式会社バイオテックジャパン) 1999.03.31、請求項１、３－４、段落００１４　& JP 11-276072 AA	１－２３
Y	T.IKENAGA et al., Enhancement of host resistance against Salmonella typhimurium in mice fed a diet supplemented with milk fermented with Lactobacillus plantarum., Milk Science (2002), Vol.51, No.1, pages 27-32	１－２３

☑ Ｃ欄の続きにも文献が列挙されている。　☐ パテントファミリーに関する別紙を参照。

＊　引用文献のカテゴリー
「Ａ」特に関連のある文献ではなく、一般的技術水準を示すもの
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「＆」同一パテントファミリー文献

国際調査を完了した日	国際調査報告の発送日
０９．０５．２００６	２３．０５．２００６

国際調査機関の名称及びあて先	特許庁審査官（権限のある職員）		
日本国特許庁（ＩＳＡ／ＪＰ） 郵便番号１００－８９１５ 東京都千代田区霞が関三丁目４番３号	村上　騎見高	４Ｎ	８８２７
	電話番号　０３－３５８１－１１０１　内線　３４８８		

C（続き）．　関連すると認められる文献

引用文献のカテゴリー＊	引用文献名　及び一部の箇所が関連するときは、その関連する箇所の表示	関連する請求の範囲の番号
Y	Y.MAO et al., Intestinal Immune Response to Oral Administration of Lactobacillus reuteri R2LC, Lactobacillus plantarum DSM 9843, Pectin and Oatbase on Methotrexate-induced Enterocolitis in Rats., Microbial Ecology in Health and Disease (1996), Vol.9, No.6, pages 261-269	1－23
Y	JP 2002-080364 A (武田食品工業株式会社) 2002.03.19,全文(ファミリーなし)	1－23
Y	Maria E. Bibas BONET et al., Optimal Effect of Lactobacillus delbrueecki subsp. bulgaricus, Among Other Lactobacilli Species, on the Number of IgA and Mast Cells Associated with the Mucosa in Immunosuppressed Mice., Food and Agricultural Immunology (1999), Vol.11, No.3, pages 259-267	1－23
Y	M.V.HERIAS et al., Immunomodulatory effects of Lactobacillus plantarum colonizing the intestine of gnotobiotic rats., Clin Exp Immunol (1999), Vol.116, No.2, pages 283-290	1－23
Y	JP 10-167972 A (武田食品工業株式会社) 1998. 06. 23, 全文(ファミリーなし)	1－23
Y	JP 2001-064174 A (武田食品工業株式会社) 2001. 03. 13, 全文(ファミリーなし)	1－23

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(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: **CLANCY, Robert** [AU/AU]; 11 High Street, Newcastle, NSW 2300 (AU). **PANG, Gerald** [AU/AU]; 4/25 Billyard Avenue, Elizabeth Bay, NSW 2011 (AU). **BORODY, Thomas** [AU/AU]; 144 Great North Road, Five Dock, NSW 2046 (AU). **DUNKLEY, Margaret** [AU/AU]; 80 Dangerfield Drive, Elermore Vale, NSW 2287 (AU). **CONWAY, Patricia, Lynne** [AU/AU]; 22 Goorawahl Avenue, La Perouse, NSW 2036 (AU).

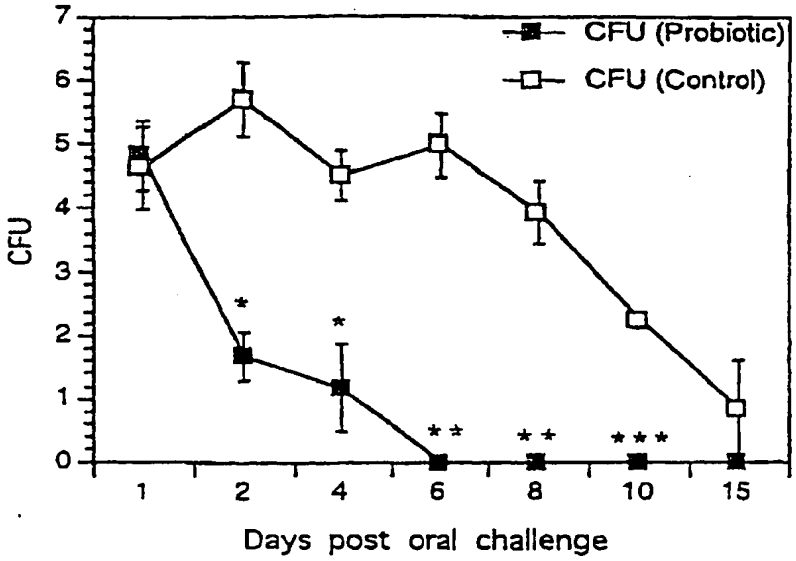
(74) Agent: **BALDWIN SHELSTON WATERS**; 60 Margaret Street, Sydney, NSW 2000 (AU).

(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European

[Continued on next page]

(54) Title: IMMUNOTHERAPY OR TREATING BACTERIAL OR VIRAL INFECTION AT MUCOSAL SURFACES WITH PROBIOTICS, AND COMPOSITIONS THEREFOR.



* P < 0.05, ** P < 0.01, *** P < 0.001

(57) Abstract: Compositions and methods for therapeutic or prophylactic treatment of disorders associated with mucosal surfaces and in particular to treatment of infectious disorders at mucosal sites by enhancing non-specific mucosal immunity, especially with probiotics such as lactobacillus or mycobacterium vaccae.

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patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

Published:

— *with international search report*

IMMUNOTHERAPY OR TREATING BACTERIAL OR VIRAL INFECTION AT MUCOSAL SURFACES WITH PROBIOTICS, AND COMPOSITIONS THEREFOR

TECHNICAL FIELD

The present invention relates to compositions and methods suitable for therapeutic or prophylactic treatment of diseases associated with mucosal surfaces and in particular to treatment of infectious disorders at mucosal site by way of enhancing non-specific mucosal immunity.

BACKGROUND ART

The last 30 years has witnessed an explosive increase in understanding of the mechanisms of mucosal protection, beginning with the recognition that mucosal immunity was partitioned from systemic immunity (with IgA as a marker), that it was driven from the gut-associated lymphoid tissue (specifically Peyer's patches), that it involved both T and B lymphocytes and that a specific recirculation of gut-derived lymphocytes between the mucosal surfaces ensured participation of all mucosal surfaces in responses generated by delivery of antigen to the Peyer's patch. Early studies focused on IgA, but gradually the key role played by T lymphocytes and the cytokines they secrete, have dominated thinking. The concept of "cytokine profiles" became important as it was shown that T cells could be characterised by the particular pattern of cytokines secreted, leading to the concept of Th1 and Th2 CD4+ve T cells. Th1 cells secreted IFN-γ while Th2 cells were characterised by IL-4 secretion. This "pattern" determined outcome and now many infection outcomes are known to be influenced by the pattern of cytokines secreted.

The above focuses on specific immunity initiated by particular antigens. The non-specific immune response "sits" on, and operates through, an array of cells and molecules which are powerful effector mechanisms operating without the specificity gained through antigen receptors.

The value in health promotion of a range of gut microbes (probiotics) taken in a variety of food or formulation forms has been recognised for some time. Claims to the value of these probiotics are mainly non-specific and without scientific support, and are largely based on clinical impression. The probiotics are thought to promote health via reconstitution of what is presumed to be beneficial normal flora. Use of probiotics for treatment of certain specific

intestinal conditions has been reported. However, the frequently exaggerated claims serve to reduce credibility rather than consolidate therapeutic benefits.

Changing patterns of proneness to mucosal disease (eg. allergy, infection) are increasingly being linked to microbe/gut mucosal "experience", in areas which include changing incidence of allergy and asthma linked to environment "sterility" and altered gut bacterial flora, and reduced mucosal infection and allergy in infants given avirulent E. coli.

There is therefore a need for improved preparations and methods for prophylaxis or therapy of mucosal disorders such as infections, allergy and the like. There is also a need for preparations and methods which can be used for prevention of reactivation of, or reinfection with, viruses and for the treatment of conditions and syndromes associated with viral infection or reinfection.

It is an object of the present invention to overcome or ameliorate at least some of the disadvantages of the prior art, or to provide a useful alternative.

SUMMARY OF THE INVENTION

The present invention is in part based on the observation that certain microorganisms, in particular lactobacilli, can prime the mucosal surfaces by inducing a particular cytokine "pattern", thus creating conditions unfavourable to microbial colonisation, and/or microbial pathogenesis, and in part on the novel demonstration of non-antigen activated cells migrating within the common mucosal system.

The microorganisms useful in the practice of the present invention may or may not have traditional probiotic effects, but they will be able to alter the cytokine pattern or balance, or induce a Th1-type cellular response. For convenience the useful microorganisms or their components may also be referred to herein as probiotics, whether or not they in fact have a probiotic effect. It is intended that this term ("probiotic") includes in its scope other adjuvant agents capable of inducing a Th1-type cellular response.

According to a first aspect there is provided a method of prophylactic or therapeutic treatment of chronic or acute infection, or of undesirable microbial colonisation, of a mucosal surface, other than intestinal mucosal surface, comprising the administration of an effective amount of a probiotic, or a probiotic-containing composition, to a subject in need thereof.

In this embodiment of the invention the mucosal surface is preferably selected from the group consisting of oral, nasopharyngeal, respiratory, gastric, reproductive and glandular. The infection or colonisation can be acute or chronic and may be bacterial, fungal or viral. It will be understood that chronic viral infection will include certain syndromes which may have origins in viral infection, such as for example chronic fatigue syndrome and the like.

According to a second aspect there is provided a method of prophylactic or therapeutic treatment of a chronic or acute disorder of mucosal surface of the respiratory tract, comprising the administration of an effective amount of a probiotic, or a probiotic-containing composition, to a subject in need thereof.

Preferably the respiratory tract mucosal surface is the upper respiratory tract mucosal surface and even more preferably it is oral or lung mucosa.

Preferably the probiotic, or a probiotic-containing composition, is administered to the gastric or to the intestinal mucosal surface.

According to a third aspect there is provided a method of prophylactic or therapeutic treatment of a chronic or acute disorder of a mucosal surface caused by disturbance in cytokine balance or lack of an appropriate T cell immune response, comprising the administration of an effective amount of a probiotic, or a probiotic-containing composition, to a subject in need thereof.

Preferably the mucosal surface is selected from the group consisting of oral, nasopharyngeal, respiratory, gastric, intestinal, reproductive and glandular.

A useful marker for assessment of cytokine balance is one or more of interferon-γ (IFN-γ), interleukin-4 (IL-4) and interleukin-12 (IL-12). However, other cytokines known as markers for either Th1 or Th2 cellular responses are also useful for this purpose.

It is preferred that the probiotic is a bacterium, for example one which can be selected from, but not limited to, lactic acid bacteria, Mycobacterium species or Bifidobacterium species. Even more preferred is the use of *Lactobacillus acidophilus* (*L. acidophilus*), *Lactobacillus fermentum (L. fermentum)* or *Mycobacterium vaccae* (*M. vaccae*), or parts thereof which are capable of inducing the Th1 cellular response. Specially preferred is *L. acidophilus*. *L. acidophilus*, *L. fermentum* or *M. vaccae* may be used live or as an inactivated preparation, as long as they are capable of inducing the Th1 response. For

preference *L. acidophilus* and *L. fermentum* is used as a live preparation. It is considered that other bacteria would also be suitable as probiotics as herein defined (whether they have probiotic effect or not), for example the well known adjuvating bacteria such as for example *L.casei, L. plantarum, L. rhamnosus, Bifidobecterium breve* and the like.

The required dosage amount will vary according to the nature of the mucosal surface disorder, whether used prophylactically or therapeutically and the type of organism or neoplasm involved. The treatment parameters as well as the required dosage can be easily assessed by those skilled in the art. The preferred dosage of the probiotic, when the probiotic is a whole live probiotic bacterium, is from about 1×10^8 to about 1×10^{12} organisms.

The probiotic may be administered in conjunction with one or more antibiotics or one or more other pharmaceutically active agents. The probiotic may be administered prior to, simultaneously with or subsequent to antibiotic therapy or therapy with other active agents.

Preferably, the mucosal surface disorder is a bacterial infection such as for example infection by Pseudomonas species, Streptococcus species, Staphylococcus species, Candida species, Helicobacter species or Haemophilus species. Even more preferred are non-typable *Haemophilus influenzae*, *Pseudomonas aeruginosa, Streptococcus pneumoniae, Staphylococcus albus, Staphylococcus aureus, Candida albicans* and/or *Helicobacter pylori.* The mucosal disorder may also be inappropriate colonisation by bacteria or other microorganisms. For example the mucosal disorder may involve chronic or acute colonisation or infection by viruses such as Epstein-Barr virus (EBV), cytomegalovirus (CMV), Herpes viruses and the like. It would be clear to those skilled in the are that the treatment of infections and/or colonisation by other viruses can also be achieved with the methods and compositions of the present invention.

Preferably, the subject in need of treatment is selected from the group consisting of individuals having high risk of infection. However, it will be recognised that the present treatments are suitably employed in prophylaxis of mucosal disorders in any subject. For example, the treatment methods of the present invention may be suitably administered to subjects exposed to a variety

of stressors which have an impact on the subjects immune status and thus predisposes them to infection. For example fatigue and/or physical stressors such as commonly encountered by athletes, predisposes these subjects to respiratory tract infections. This seems to be caused by impairment in the immune status of mucosal surfaces, in particular the secretion of IgA.

Administration of the probiotic preparations, such as those described herein, prior to, during and/or after exercise or training may restore their immune status, particularly that of the mucosal surfaces of the respiratory tract, thus combating or preventing infection.

Preferably, the probiotic or probiotic-containing composition is in tablet or capsule form. However, it will be clear to those skilled in the art that the probiotic composition may be in a liquid or other forms of solid preparations and may also be present in a food source such as a yoghurt or other dairy product, or similar non-dairy products based for example on soy..

The treatment may involve administration of a probiotic, or of a probiotic-containing composition, to a site which is distal to the mucosal surface having the disorder. For example the probiotic, or the probiotic-containing composition, can be administered to the intestinal mucosal surface in the treatment of nasopharyngeal, gastric or upper respiratory tract mucosal infection.

According to a fourth aspect there is provided a method of altering cytokine balance at a mucosal surface in a subject comprising the administration of an effective amount of a probiotic, or of a probiotic-containing composition, to a subject in need thereof.

According to a fifth aspect there is provided a method of inducing a Th1 cellular immune response at a mucosal surface in a subject comprising the administration of an effective amount of a probiotic, or of a probiotic-containing composition, to a subject in need thereof.

According to a sixth aspect there is provided a method of enhancing the secretion of interferon-γ at a mucosal surface in a subject comprising the administration of an effective amount of a probiotic, or of a probiotic-containing composition, to a subject in need thereof.

According to a seventh aspect there is provided a method of reducing the secretion of interleukin-4 at a mucosal surface in a subject comprising the

administration of an effective amount of a probiotic, or of a probiotic-containing composition, to a subject in need thereof.

According to an eighth aspect there is provided a method of restoring normal cytokine balance at a mucosal surface in a subject comprising the administration of an effective amount of a probiotic, or of a probiotic-containing composition, to a subject in need thereof.

According to a ninth aspect there is provided a method of priming mucosal surface for immunotherapy comprising the administration of an effective amount of a probiotic, or of a probiotic-containing composition, to a subject in need thereof.

Preferably the immunotherapy consists in administration of a therapeutic or prophylactic vaccine. Also preferred is a procedure whereby the probiotic, or the probiotic-containing composition, is administered before or simultaneously with the vaccine. The administration of the probiotic, or of the probiotic-containing composition, may be continued for a period after the vaccine has been administered.

According to a tenth aspect there is provided a pharmaceutical composition suitable for prophylactic or therapeutic treatment of chronic or acute disorder of a mucosal surface comprising an effective amount of a probiotic, or of a probiotic-containing composition.

It is preferred that the probiotic is a bacterium, for example one which can be selected from, but not limited to, lactic acid bacteria, Mycobacterium species or Bifidobacterium species. Even more preferred is the use of *Lactobacillus acidophilus* (*L. acidophilus*), *Lactobacillus fermentum (L. fermentum)* or *Mycobacterium vaccae* (*M. vaccae*), or parts thereof which are capable of inducing the Th1 cellular response. Specially preferred is *L. acidophilus*. *L. acidophilus*, *L. fermentum* or *M. vaccae* may be used live or as an inactivated preparation, as long as they are capable of inducing the Th1 response. For preference *L. acidophilus* and *L. fermentum* is used as a live preparation. It is considered that other bacteria would also be suitable as probiotics as herein defined (whether they have probiotic effect or not), for example the well known adjuvating bacteria such as for example *L.casei, L. Plantarum, L. rhamnosus, Bifidobecterium breve* and the like.

Preferably the pharmaceutical composition includes viable organisms, however it will be understood that killed organisms may also be used. Further, single organism preparations or preparations containing multiple organisms are envisaged. The organism(s) can be either intact or disrupted.

The preferred content of the probiotic, when the probiotic is a whole live probiotic bacterium, is one that will deliver a dosage from about 1×10^8 to about 1×10^{12} organisms.

The preferred formulation of the composition is in a solid dosage form, such as for example tablet or capsule. It will be understood however that it may be formulated in the form of a food product, for example soy-based or dairy-based product.

According to an eleventh aspect there is provided a method of prophylactic or therapeutic treatment of a symptom and/or syndrome associated with chronic or acute infection, or with undesirable microbial colonisation or reactivation, of a mucosal surface, comprising the administration of an effective amount of an agent capable of non-specific activation of the common mucosal system to a subject in need thereof.

Preferably the symptom and/or syndrome to be treated is chronic fatigue syndrome.

The preferred agent is a probiotic, or a probiotic-containing composition. Also preferred is that the probiotic is, or the probiotic-containing composition comprises, viable intact organisms.

Preferably the subject to be treated is selected from the group consisting of an EBV positive athlete, a subject with sudden onset CFS, a subject with protracted fatigue following exercise, a subject with low level of salivary IgA or IgA1 and a subject with documented infectious mononucleosis.

BRIEF DESCRIPTION OF FIGURES

Figure 1: IL-4 and IFN-γ production following feeding with *Lactobacillus acidophilus*

Figure 2: Secretion of NO into saliva of animals fed *Lactobacillus acidophilus* following challenge with *Candida albicans*

Figure 3: Effect of probiotic on IL-12 production following challenge with *Candida albicans*

Figure 4: Effect of probiotic on IL-4 production following challenge with *Candida albicans*

Figure 5: Resistant to infection in animals fed *L. acidophilus.*

Figure 6: Effect of probiotic on salivary IFN-γ following challenge with *Candida albicans*

Figure 7: Effect of probiotic on cervical lymph node IFN-γ following challenge with *Candida albicans*

Figure 8: Salivary IFN-γ following challenge with *C albicans.*

Figure 9: Effect of treatment with L-MLNA on clearance of *C.albicans* in mice

DESCRIPTIOON OF THE PREFERRED EMBODIMENTS

The current invention is about the manipulation of cells and molecules that participate in both these systems, to "prime" mucosal surfaces to produce Th1 cytokines in response to "danger". The concept that is developed is that certain bacteria, specially lactobacilli, populate the gut of subjects exposed to high density pathogens. Our studies have shown that these bacteria influence the cytokine pattern towards a Th1 response. The activated and IFN-γ producing T cells populate the mucosal surfaces and their regional lymph nodes, setting up a containing armament awaiting "danger".

The methods and compositions of the present invention can also be used effectively in the treatment of acute and chronic viral infections. In particular the treatment of chronic Epstein-Barr virus (EBV), cytomegalovirus (CMV) and other herpes-type virus infection, which are ubiquitous in the population and are associated with numerous symptoms and diseases, are envisaged as particularly useful applications. Also envisaged as a useful application of the compositions and methods of the present invention is the treatment of disorders which may have origins in or are triggered by viral infections which are prevalent in the population and which can be debilitating but have no known or no well defined treatment protocol, such as for example chronic fatigue syndrome ("CFS").

Substantial number of patients currently diagnosed as "CFS" reflect repeatedly activated EBV (or similar herpes group virus infection), due to

impaired mucosal immunity. This can be monitored through measurement of salivary IgA1 or INF-γ, or PCR detected free virus. Characteristic (but not limiting) clinical features of those likely to respond to probiotic therapy includes onset of "viral illness", leaving a state of chronic relapsing fatigue. Acute deterioration occurs with "infection", usually noted as a sore throat, or excessive exercise.

Viral infection need not cause immediately recognisable symptoms and may be dormant in otherwise fit and healthy subjects for a significant periods of time, but may reactivate once a trigger is provided. For example, in athletes undergoing intensive training or exercise impaired performance may be caused by reduced mucosal containment of EBV, which leads to reactivation and excretion of virus, which in turn leads to impaired performance and/or fatigue. Thus, athletes who are EBV positive and have impaired performances, specially if low salivary IgA or IgA1 is detected (for example less than 50 mg/100ml), could benefit from treatment with preparations of the present invention.

Of course the preparations and methods of the present invention can also be applied to other subjects, for example patients diagnosed as "CFS", where a virus (usually EBV but can be CMV, Ross River virus and the like) is reactivated and the fatigue is particularly initiated by exercise. However, any patient with documented infectious mononucleosis not getting better within several weeks and left with fatigue, any patient with protracted fatigue following clinical (± serological) evidence of viral illness or subjects with sudden onset CFS, with recurrent sore throats, with significant exacerbation of fatigue following exercise, or with low level of salivary IgA or IgA1 (eg. less than 50 mg/100ml), could benefit from treatment with the preparations of the present invention.

It is envisaged that the administration of the probiotic or compositions containing the probiotic, will also assist in the treatment of symptoms and/or disorders described above in relation to viral infections.

The probiotics have the effect on mucosal surfaces distal to the site of administration of the probiotic. Thus, in broad terms the present invention is concerned with a probiotic product, in particular a product which is or includes lactobacilli, but may include other bacteria or combinations of bacteria, or

indeed other adjuvants capable of inducing a Th1-type cellular response, which can be administered as a bolus or preferably regularly fed to maintain optimal mucosal protection of all mucosal surfaces through priming of mucosal T cells or maintaining the primed state of the cells. Such T cells contain INFγ and can be triggered to release INF-γ by non specific mechanisms. Therefore, other agents capable of activating non-specifically the common mucosal system may also be advantageously used in the preparations and methods of the present invention.

Further, the probiotics of the present invention may be used in conjunction with other treatments, to enhance or assist in their efficacy. For example, approximately 20% of patients treated with antibiotics for H. pylori infection fail to eradicate the organism. This resistance to antibiotic therapy may be due to a shift towards a Th0 response (ie, less IFN-γ and more IL-4). Administration of a probiotic prior to, in conjunction with or subsequent to antibiotic therapy can be beneficial by switching back to a more dominant Th1 response and thus supplementing or assisting the antibiotic therapy to eradicate the organism in such patients.

Administration of probiotic can therefore be used for prevention or therapy of mucosal infections, colonisation of mucosal surfaces with abnormal or inappropriate organisms and reinfection with or reactivation of viruses.

The invention will now be described more particularly with reference to non-limiting examples.

EXAMPLES

Example 1: Effect of probiotic bacteria on Th1/Th2 cytokine response

To determine whether probiotic bacteria down-regulate Th2 and up-regulate Th1 cytokine response, C57/Bl6 mice were fed intragastrically using a feeding needle, various numbers of *Lactobacillus acidophilus* (obtained from University of New South Wales, School of Microbiology and Immunology Culture Collection, Sydney, Australia) on consecutive days for 2 weeks after which they were sensitised with 8 μg of ovalbumin (OVA) and aluminium hydroxide in 0.2 mL phosphate-buffered saline administered by peritoneal injection. The mice

were further fed ten times with *L. acidophilus* every two days for two weeks before they were sacrificed. Lymphocytes were isolated by teasing spleens through a sieve, washed with PBS, and resuspended at 10 x 10^6 One mL aliquots of the cell suspension were dispensed into wells of a 24-well flat-bottomed microtitre plate and stimulated with OVA (5 μg/mL). After incubation for 4 days the supernatants were collected and assayed for IL-4 and IFN-γ production by standard ELISA techniques using IL-4 or IFN-γ monoclonal antibody pairs.

Briefly, wells of a 24-well microtitre plate were coated with a capture anti-IL-4 antibody. After incubation at room temperature for 1 hr, the wells were washed and biotinylated anti-IL-4 antibody was added to each well. Following incubation for a further 1 hr, the wells were washed and strepavidin-peroxidase conjugate was added to each well. After incubation for 30 mins, the wells were washed and then TMB substrate was added. The colour development was read at 450/620 nm in an ELISA plate reader. The level of IL-4 in unknown samples was quantitated by interpolation using a standard curve. A similar procedure was used for measurement of IFN-γ.

The results shown in Fig. 1 A and B demonstrate that feeding *L. acidophilus* resulted in the suppression of IL-4 production in dose-dependent manner (Fig. 1A) whereas the production of IFN-γ was enhanced (Fig 1B).

Example 2: Effect of probiotic bacteria on nitric oxide (NO) secretion in saliva

DBA/2 mice (n= 3 to 5 per group) were fed *Lactobacillus acidophilus* and then challenged with *Candida albicans* as described in Example 3 below. At various times following oral infusion, saliva was collected after injection with pilocarpine to stimulate saliva flow. The concentration of NO in saliva was determined by the Griess reaction according to the method by Gree et al (1982) Anal. Biochem 126:131-138.

The data demonstrate that secretion of NO into saliva of animals fed *Lactobacillus acidophilus* (oral administration of 5x10^9 CFU) was increased early

after challenge with *Candida albicans* but was significantly reduced at a later stage (Figure 2).

Example 3 Resistance to *C. albicans* following feeding with probiotic bacteria

DBA/2 mice H2d male mice (n = 3-5 per group) were fed intragastrically every 2 days for 2 weeks with 5x 10^9 *L. acidophilus* in 0.2mL PBS. One day after the last feed mice were challenged with 10^8 *Candida albicans* blastospores by swabbing the oral cavity using a fine-tip sterile swab. At various days after infection the number of *C. albicans* in the oral mucosa was determined. The oral cavity (cheek, tongue and soft palate) was completely swabbed using a fine-tipped cotton swab. The cotton swab was placed in 1 mL PBS and the yeast cells were resuspended by mixing in a vortex mixer before culture of serial 10-fold dilutions on Sabourand dextrose agar supplemented with chloramphenicol at 37°C. The results were expressed as CFU/mL (Figure 5)

Mice fed L. acidophilus were more resistant to infection at all time points compared to control mice fed PBS. By day 6 the yeasts were almost completely cleared from mice fed *L. acidophilus*.

Example 4: IL-4, IFN-γ and IL-12 production

A further study on responses to *Candida albicans* infection was conducted in the model described in Example 3.

Candida antigen was prepared from freshly cultured *Candida albicans* by sonication in a MSE Soniprep. The sonicate was centrifuged for 10 min at 2000g after which time the supernatant was collected and dialysed against PBS. After protein estimation, the solution was filtered sterilised and stored at -20°C until needed.

A single cell suspension of cervical lymph node (CLN) cells was prepared by teasing lymph node tissue through a sieve. The cells were collected in RPMI 1640 medium supplemented with 10% foetal calf serum (FCS), washed twice by centrifugation and cultured at 4x10^6 cells per well in the presence of 2.5μg/mL of Candida antigen in a 24-well plate for 3 days.

The culture supernatants were collected and assayed for IL-4, IL-12 and IFN-γ by ELISA using matched antibody pairs and recombinant cytokines as

standards (Pharmingen, San Diego, CA). The sensitivity of the cytokine ELISA was 31pg/mL and the results were expressed in net amounts from which the background was subtracted.

Samples of saliva were obtained according to the procedure described in example 2.

The results of IL-4 and IL-12 study are shown in Figure 3 (IL-12) and Figure 4 (IL-4).

The results of the IFN-γ study are shown in Figures 6 (salivary IFN-γ) and 7 (cervical lymph node IFN-γ).

The control data shows a small poorly sustained response to oral infection with Candida albicans, with no detectable IFN-γ until day 6 of the infection. The data in mice fed *L. acidophilus* indicates that there is no IFN-γ response in uninfected mice but that there is an early response (day 2) when mice were challenged with *C. albicans*. Further, there was a more sustained IFN-γ response after challenge with *C. albicans* than in the controls not fed *L. acidophilus*. This primary effect can still be seen in the nodes at day 15 and in saliva at day 8.

Not wishing to be bound by any particular mechanism of action, it seems that the mucosal surfaces are primed by the administration of Lactobacillus to secrete IFN-γ following a challenge and in this model IFN-γ operates to prevent conversion of the pathogen, *Candida albicans*, to the mycelial (invasive) form of the fungus and enhances cellular immunity and nitric oxide production.

Example 5. Role of IFN-γ and NO in resistance to infection

To identify the immune parameters of protection, the levels of IFN-γ were determined at various times following infection with *C. albicans*. As shown in Fig 8, there was a marked increase in the levels of IFN-γ in saliva immediately and on day 1 after challenge with *C albicans* compared with unimmunised mice. The levels were sustained over 15 days with significantly higher levels detected on day 10 and day 15 compared with those in unimmunised mice. Since nitric oxide production is associated with host defence in parasitic infection, quantitation of NO was performed following infection in two mouse strains sharing the same H2d MHC haplotype. In this experiment mice were infected with *C albicans* and

then followed by ip injection with an inhibitor of NO synthase, by injecting NG - monomethyl-L-arginine monoacetate (MNLA) daily for 3 days after which time the clearance rate of yeasts was determined. As shown in Fig 9, mice treated with MNLA had delayed clearance of yeasts at various time points in the two mouse strains compared with untreated mice, indicating that reduction in NO production is associated with resistance.

Example 6: Enhanced clearance of non-typable *H influenzae* from the lungs of rats fed *L acidophilus*

DA rats (200- 250 gm, 8-10 weeks old, Animal Resource Centre, Perth, WA) were fed intragastrically *L acidophilus* in 0.75 mL PBS (2.5×10^{10} per rat) or PBS alone every 2 days for 7 days at which time the rats were immunised with formalin-killed *H influenzae* (5×10^{9} per rat) administered intralumenally (see groups below). Rats continued to be fed every 2 days for 2 weeks and then boosted with 50 μL of formalin killed *H influenzae* (5×10^{8} per rat) administered by the intratracheal route. After further feeding with *L acidophilus* for a further 7 days, the rats were challenged with 50 μL of live 5×10^{8} *H influenzae* in the lung.

Immunisation groups (5 rats/group):

1 PBS (intralumenal)

2 *H influenzae* (intralumenal) + *H influenzae* (intra-tracheal boost)

3 PBS (intralumenal) + *L. acidophilus* fed every 2 days

4 *H influenzae* (intralumenal) + *H influenzae* (intratracheal boost) + *L. acidophilus* fed every 2 days

After 4 hrs post challenge, the rats were sacrificed and the levels of colonisation in the lung was examined in bronchial lavage (BAL) and lung homogenate (LH). The level of clearance was determined by a plating serial 10-fold dilutions of the lavage fluid or lung homogenate onto chocolate agar plates. The results were expressed as number of colony forming units (CFU.

The results are shown in Table 1. Rats fed *L acidophilus* and immunised with killed *H influenzae* were more resistance to infection *H influenzae* in the lungs. Furthermore, rats fed orally with repeated doses of *L acidophilus* were more resistant to infection than rats given a single bolus of *L*

acidophilus, suggesting that enhanced clearance may be due to increased colonisation in the gut with *L acidophilus* following repeated feeding.

Table 1 Increased clearance of *H influenzae* in the lungs of rats fed *L acidophilus*

Groups	BAL (CFU/mL x 10^6)	LH (CFU/mL x 10^6)
PBS	26 ± 7.5	90.2 ± 29
H influenzae + *H influenzae* boost	4.6 ± 3.0	4.9 ± 1.2
PBS + *L. acidophilus*	0.7 ± 0.3	12.5 ± 5.7
H influenzae + *H influenzae* boost + *L. acidophilus*	0.19 ± 0.1	3.2 ± 2.1

Example 7. Effect of *Lactobacillus acidophilus* (VRI 011) on translocation of *Salmonella typhimurium* in mice following immunisation with heat killed Salmonella vaccine.

Translocation of gram-negative bacteria across the gut epithelium can occur especially in subjects following post-operative surgery or gastrointestinal infection. Left untreated it can lead to endotoxiemia. In this example, the effect of feeding *L acidophilus* on the translocation of gut pathogen *Salmonella typhimurium* is examined.

Female BALB/c mice 6-8 weeks old were obtained from the Animal Resource Centre, Perth, WA. *Lactobacillus acidophilus* strain (VRI 011) and the pathogen *Salmonella tyhimurium* were obtained from the School of Microbiology and Immunology Type Culture Collection, University of New South Wales.

Mice were given 5 doses of *L acidophilus* (VRI 011) (1 x 10^8 cfu per dose) or PBS over a 2 week period after which time mice were immunised intragastrically with a killed oral Salmonella vaccine. Two weeks after immunisation, the mice were sacrificed and the spleens collected for enumeration of *L acidophilus* and *S tyhimurium*.

Mice fed *L acidophilus* (VRI 011) before or simulatneously with a killed oral *S typhimurium* vaccine have better protection against translocation of *S tyhpimuirum* following live challenge via the oral route compared with mice fed PBS (Table 1). Immunisation with a killed oral vaccine led to decreased translocation of *S typhimurium* to the spleen but it was further enhanced by feeding *L acidophilus*. Thus translocation of pathogenic bacteria through the gut epithelium can be prevented by oral administration of probiotic.

Example 8: Effect of *Lactobacillus fermentum* on translocation of pathogenic *S tyhphimurium* from the gut of mice vaccinated with killed oral Salmonella vaccine

BALB/c female mice (6-8 weeks old) were fed 10^8 *L fermentum* VRI012 (obtained from University of New South Wales, School of Microbiology and Immunology Culture Collection, Sydney, Australia) before or co-administered with 10^8 killed *S typhimurium* vaccine four times over a 2 week period. Two weeks after oral administration, the mice were challenged with live *S typhimurium* administered by the oral route. After sacrifice, the level of colonisation with *S typhimurium* in the spleen was determined. Table 2 showed that low levels of translocation occurred in mice fed *L fermentum* prior to immunisation or co-administered with *S typhimurium* compared with mice fed PBS.

Table 2 *L fermentum* VRI012 enhanced the inhibition of translocation of *S typhimurium* in mice immunsed with killed *S typhimurium* vaccine.

Group treatment	**Log CFU/g wet wt of spleed (± SEM)**
PBS (Control)	10.12 ± 0.54
Killed *S typhimurium*	5.5 ± 0.27 $p < 0.05$ vs Control
L fermentum KLD (before)	3.8 ± 0.12 $p < 0.05$ vs Control
L fermentum KLD (co-administered)	4.5 ± 0.22 $p < 0.05$ vs Control

Example 9. Effect of *L. fermentum* on clearance of NTHi bacteria

Groups of 6 male, specific pathogen free, dark agouti (DA) rats were given a single dose of PBS or live *L. fermentum* (2.5×10^{10}) directly into the duodenal lumen after exposure of the intestine by laparotomy. Twenty-one days later the rats were infected intratracheally with 5×10^8 live Non-typeable *Haemophilus influenzae* (NTHi). Four hours later the rats were killed by pentobarbitone overdose. The lungs were lavaged with 10 mL PBS to obtain broncho-alveolar lavage fluid (BAL). The lavaged lungs were then homogenised in 10mL of PBS. Twenty microlitres of ten-fold serial dilutions of BAL and lung homogenate (LH) were plated on chocolate agar plates and incubated in a 10% CO2 incubator at 37°C overnight. Colonies were counted and the number of live bacteria in the BAL and LH preparations determined. The recovery of live NTHi bacteria, presented as colony forming units (CFU), was as follows:

Table 3.

Rats dosed with:	BAL CFU (10^6)	LH (10^6)
PBS	3.67 ± 1.30	103.5 ± 34.2
L. fermentum	0.49 ± 0.19	0.82 ± 0.37 p = 0.017*

* When compared to PBS group

These data show that a single dose of *L. fermentum* delivered to the gut causes enhanced clearance of a subsequent acute NTHi respiratory infection.

Example 10. Effect of *L acidophilus* on clearance of *H. pylori*

Two groups of 5 female C57BL/6 mice were infected with *H. pylori* (Sydney strain 1) by administration of approx. 10^9 live *H. pylori* on three consecutive days (days 1,2 and 3) by gavage. On days 28, 30, 32, 35, 37 and 39 mice were dosed by gavage with live *L. acidophilus* (5×10^9 in 0.2 mL water) or water (control group).

Twenty-one days later (day 50) mice were killed by pentobarbitone overdose (administered intra-peritoneally) and their stomachs removed. The stomach was dissected into two equal halves and one half was placed in 1mL of deionised water and homogenised. Serial 10-fold dilutions of stomach homogenate were plated out on chocolate agar plates containing fungizone, vancomycin, bacitracin and nalidixic acid. After incubation for 3 days at 37°C under microaerophilic conditions, the colonies were counted and the total

number of live *H. pylori* in the original stomach homogenate determined. The results for recovery of live *H. pylori* in the stomach, expressed as colony-forming units (CFU), are shown in the following table.

Table 4:

Mice dosed with:	Number of H. pylori in stomach homogenate (10^4 CFU)
Deionised water	252 ± 44
L. acidophilus in water	105 ± 14

The two groups are significantly different (P = 0.013)

These results show that mice dosed with *L. acidophilus* have a lower level of *H. pylori* infection of the stomach than do control mice. This is an example of a therapeutic effect of *L. acidophilus* on a pre-established *H. pylori* infection.

Example 11: Anti-bacterial therapy (Metronidazole) of Helicobacter pylori infection in mice pre-treated with probiotic

H. pylori infected mice (5 per group) were treated with *Lactobacillus acidophilus* ($1x10^9$ per animal) by oral feeding 3 times a week for 2 weeks prior to treatment with Metronidazole (0.08mg/animal) for 1 week.

The following results demonstrate that highly significant eradication of bacteria from the stomach was noted in mice pre-treated with probiotic and followed by antibiotic therapy (70%, p=0.009), those treated with probiotic alone (58%, p=0.013), and those treatment with metronidazole alone (55%, p,0.025), when compared with the saline-treated control group.

Table 6:

Treatment Group	*H. pylori* colonisation (x 10^5 CFU/animal)	% eradication
Saline	25 ± 4.3	0
L acidophilus	10.5 ± 1.4	58[a]
Metronidazole	11.4 ± 2.5	55[b]
L acidophilus + Metronidazole	7.5 ± 2.6	70[c]

a, p<0.01; b, p<0.02; c, p<0.005 compared with control values

The above examples demonstrate that probiotic bacteria, by driving a particular cytokine balance (from gut derived but mucosally re-located T cells), create an environment less favourable for particular microbial growth.

Although the present invention has been described with reference to preferred embodiments it will be understood that variations in keeping with the inventive concept described herein are also contemplated.

CLAIMS DEFINING THE INVENTION ARE AS FOLLOWS:-

1 A method of prophylactic or therapeutic treatment of chronic or acute infection, or of undesirable microbial colonisation, of a mucosal surface, other than intestinal mucosal surface, comprising the administration of an effective amount of a probiotic as herein defined, or of a probiotic-containing composition, to a subject in need thereof.

2 A method according to claim 1 wherein the mucosal surface is selected from the group consisting of oral, nasopharyngeal, respiratory, gastric, reproductive and glandular.

3 A method of prophylactic or therapeutic treatment of a chronic or acute disorder of mucosal surface of the respiratory tract, comprising the administration of an effective amount of a probiotic as herein defined, or of a probiotic-containing composition, to a subject in need thereof.

4 A method according to claim 3, wherein the mucosal surface is oral mucosa.

5 A method according to claim 3, wherein the mucosal surface is lung mucosa.

6 A method according to any one of the preceding claims, wherein the probiotic as herein defined, or the probiotic-containing composition, is administered to the gastric or to the intestinal mucosal surface.

7 A method of prophylactic or therapeutic treatment of a chronic or acute disorder of a mucosal surface caused by disturbance in cytokine balance or lack of an appropriate T cell immune response, comprising the administration of an effective amount of probiotic bacteria, or a probiotic bacteria-containing composition, to a subject in need thereof.

8 A method according to claim 6 or claim 7 wherein the mucosal surface is selected from the group consisting of oral, nasopharyngeal, respiratory, gastric, intestinal, reproductive and glandular.

9 A method according to any one of claims 3 to 8, wherein the disorder is an infection or inappropriate microbial colonisation.

10 A method according to claim 9 wherein the infection is a bacterial infection caused by one or more bacteria selected from the group consisting of

Pseudomonas species, Streptococcus species, Staphylococcus species, Candida species, Helicobacter species and Haemophilus species.

11 A method according to claim 10 wherein the one or more bacteria are selected from the group consisting of non-typable *Haemophilus influenzae*, *Pseudomonas aeruginosa, Streptococcus pneumoniae, Staphylococcus albus, Staphylococcus aureus, Candida albicans* and *Helicobacter pylori.*

12. A method according to claim 9, wherein the disorder is an infection or inappropriate colonisation by a virus.

13. A method according to claim 12, wherein the virus is selected from the group consisting of Epstein-Barr virus (EBV), cytomegalovirus (CMV), Ross River virus (RRV), herpes-type virus and the like.

14. A method according to claim 9 or claim 12, wherein the disorder is chronic fatigue syndrome (CFS).

15 A method according to any one of claims 7 to 14 wherein the cytokine is interferon-γ, interleukin-4 and/or interleukin-12.

16 A method according to any one of claims 9 to 15, wherein the appropriate T cell immune response is Th1 response.

17 A method according to any one of claims 1 to 16, wherein the probiotic as herein defined is, or probiotic-containing composition contains, a *Lactobacillus* or a *Mycobacterium* species.

18 A method according to claim 17, wherein the probiotic as herein defined is, or probiotic-containing composition contains, *Lactobacillus acidophilus*, *Lactobacillus fermentum*, *Lactobacillus casei* and/or *Mycobacterium vaccae.*

19 A method according to claim 17 or claim 18 wherein the probiotic as herein defined, or probiotic-containing composition, includes viable organisms.

20 A method according to any one of claims 1 to 19, wherein the effective amount is in the range of about 10^8 to 10^{12} CFU

21 A method according to claim 20 wherein the effective amount is about 10^{11} CFU.

22 A method according to any one of claims 1 to 21, wherein the probiotic as herein defined or probiotic-containing composition is in a solid dosage form.

23 A method according to claim 22, wherein the dosage form is a tablet or a capsule.

24 A method according to any one of claims 1 to 23, further comprising the administration of one or more pharmaceutically active agents.

25 A method according to claim 24, where the one or more pharmaceutically active agents is an antibiotic.

26 A method according to claim 24 or claim 25, wherein the probiotic as herein defined, or the probiotic-containing composition, is administered prior to, simultaneously with or subsequent to one or more pharmaceutically active agents.

27 A method according to any one of claims 1 to 26, wherein the site of administration of the probiotic as herein defined, or of the probiotic-containing composition, is distal to the mucosal surface having the disorder.

28 A method according to claim 27, wherein the probiotic as herein defined, or the probiotic-containing composition, is administered to the intestinal mucosal surface in the treatment of oral, nasopharyngeal, gastric, respiratory tract, reproductive tract or glandular mucosal infection.

29 A method of altering cytokine balance at a mucosal surface in a subject comprising the administration of an effective amount of a probiotic as herein defined, or of a probiotic-containing composition, to a subject in need thereof.

30 A method of inducing a Th1 cellular immune response at a mucosal surface in a subject comprising the administration of an effective amount of a probiotic as herein defined, or of a probiotic-containing composition, to a subject in need thereof.

31 A method of enhancing the secretion of interferon-γ at a mucosal surface in a subject comprising the administration of an effective amount of a probiotic as herein defined, or of a probiotic-containing composition, to a subject in need thereof.

32 A method of reducing the secretion of interleukin-4 at a mucosal surface in a subject comprising the administration of an effective amount of a probiotic as herein defined, or of a probiotic-containing composition, to a subject in need thereof.

33 A method of restoring normal cytokine balance at a mucosal surface in a subject comprising the administration of an effective amount of a probiotic as

herein defined, or of a probiotic-containing composition, to a subject in need thereof.

34 A method of priming mucosal surface for immunotherapy comprising the administration of an effective amount of a probiotic as herein defined, or of a probiotic-containing composition, to a subject in need thereof.

35 A method according to claim 34, wherein immunotherapy consists in administration of a therapeutic or prophylactic vaccine.

36 A method according to claim 34 or claim 35, wherein the probiotic as herein defined, or the probiotic-containing composition, is administered before or simultaneously with the vaccine.

37 A method according to claim 35 or claim 36, wherein the probiotic as herein defined, or the probiotic-containing composition, is additionally administered after the vaccine.

38 A pharmaceutical composition suitable for prophylactic or therapeutic treatment of chronic or acute disorder of a mucosal surface comprising an effective amount of a probiotic as herein defined, or of a probiotic-containing composition.

39 A pharmaceutical composition according to claim 38, wherein the probiotic as herein defined is, or probiotic-containing composition contains, a *Lactobacillus* or a *Mycobacterium* species.

40 A pharmaceutical composition according to claim 39, wherein the probiotic as herein defined is, or probiotic-containing composition contains, *Lactobacillus acidophilus*, *Lactobacillus fermentum*, *Lactobacillus casei* and/or *Mycobacterium vaccae*.

41 A pharmaceutical composition according to any one of claims 38 to 40, wherein the probiotic as herein defined, or probiotic-containing composition includes, viable organisms.

42 A pharmaceutical composition according to any one of claims 38 to 41, wherein the composition contains about 10^8 to about 10^{12} CFU

43 A pharmaceutical composition according to claim 42 wherein the composition contains about 10^{11} CFU.

44 A pharmaceutical composition according to any one of claims 38 to 43, wherein the pharmaceutical composition is in a solid dosage form.

45 A pharmaceutical composition according to claim 44, wherein the dosage form is a tablet or a capsule.

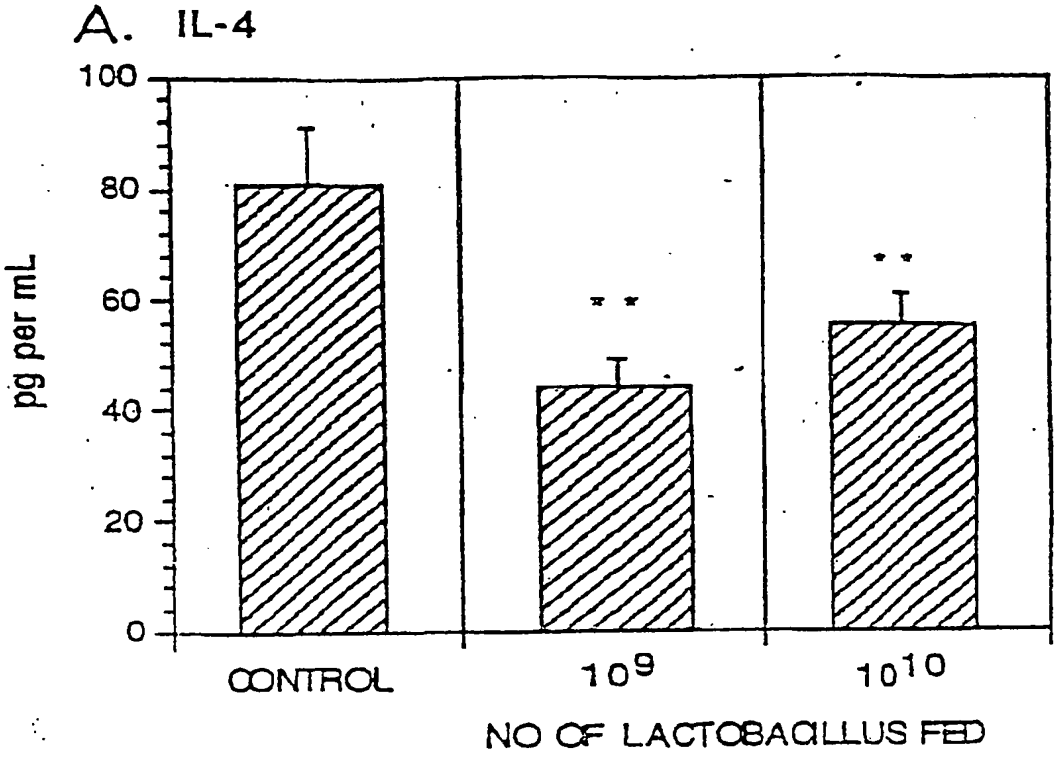
46 Method of prophylactic or therapeutic treatment of a symptom and/or syndrome associated with chronic or acute infection, or with undesirable microbial colonisation or reactivation, of a mucosal surface, comprising the administration of an effective amount of an agent capable of non-specific activation of the common mucosal system to a subject in need thereof.

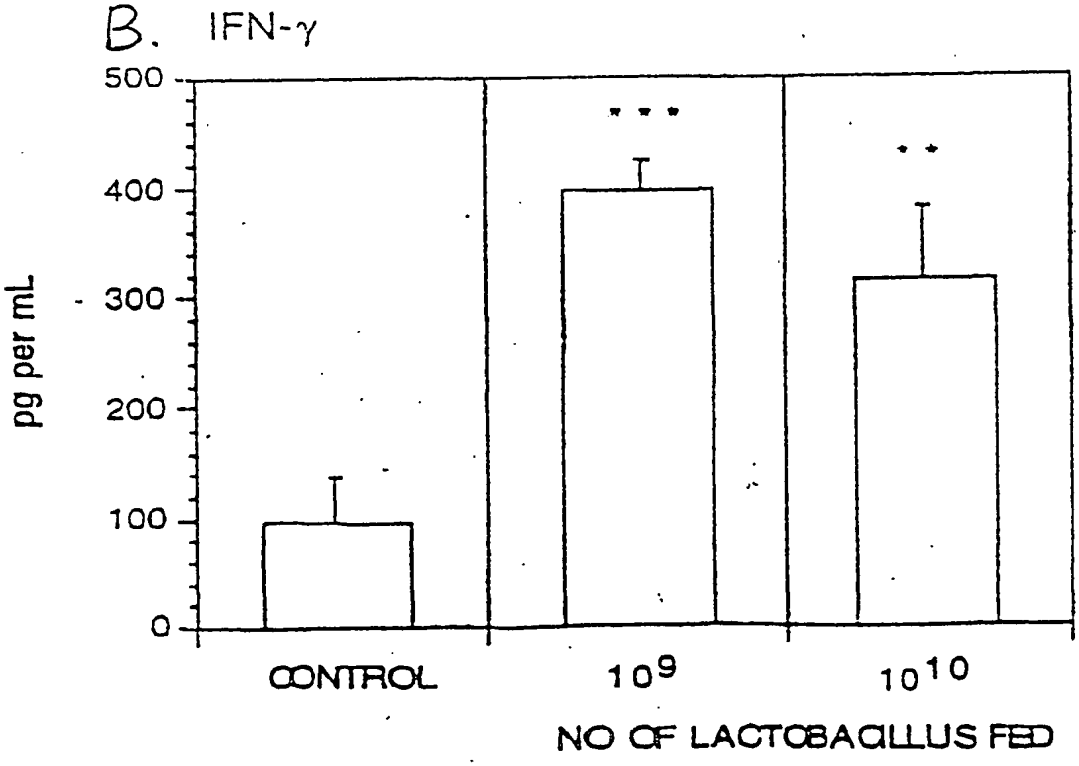
47. A method according to claim 46, wherein the symptom and/or syndrome is chronic fatigue syndrome.

48. A method according to claim 46 or claim 47, wherein the agent is a probiotic as herein defined, or a probiotic-containing composition.

49. A method according to claim 48, wherein the probiotic as herein defined is, or the probiotic-containing composition comprises, viable intact organisms.

50. A method according to any one of claims 46 to 49, wherein the subject is selected from the group consisting of an EBV positive athlete, a subject with sudden onset CFS, a subject with protracted fatigue following exercise, a subject with low level of salivary IgA or IgA1 and a subject with documented infectious mononucleosis.





**, $p < 0.01$ ***, $p < 0.001$

Figure 1

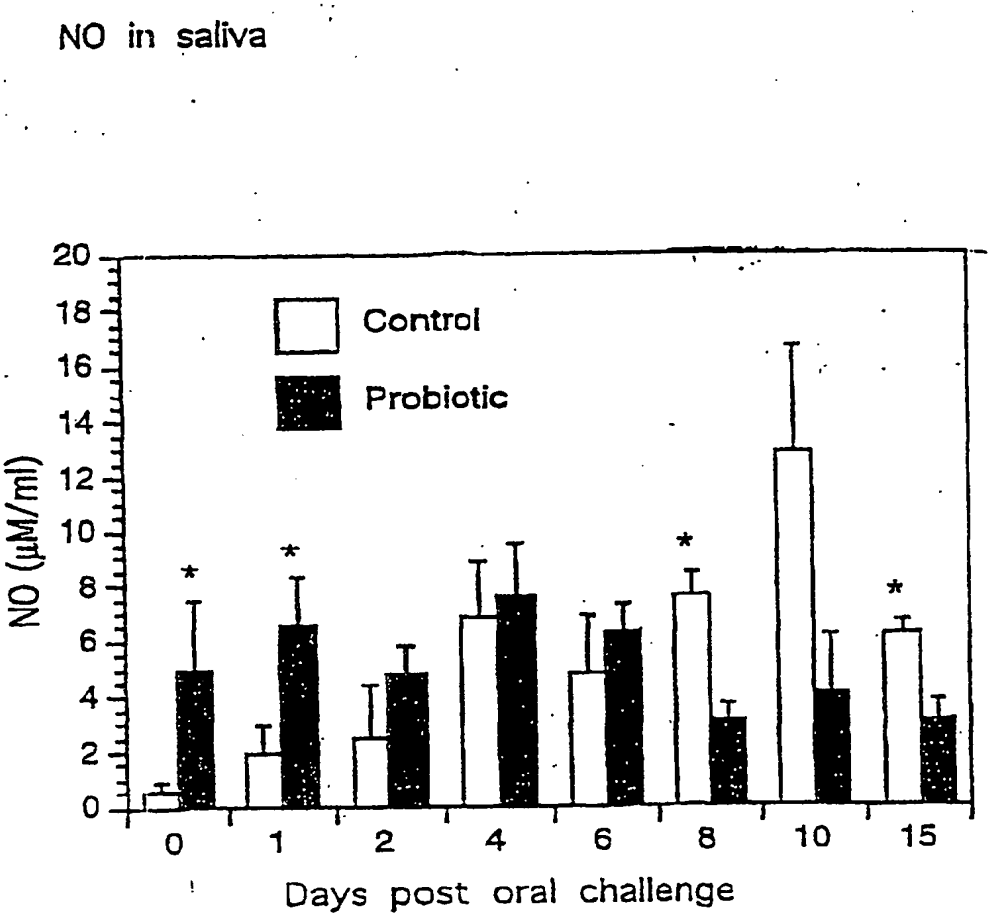

NO in saliva
NO (µM/ml)
20
18
16
14
12
10
8
6
4
2
0
Control
Probiotic
*
*
*
*
0
1
2
4
6
8
10
15
Days post oral challenge

Figure 2

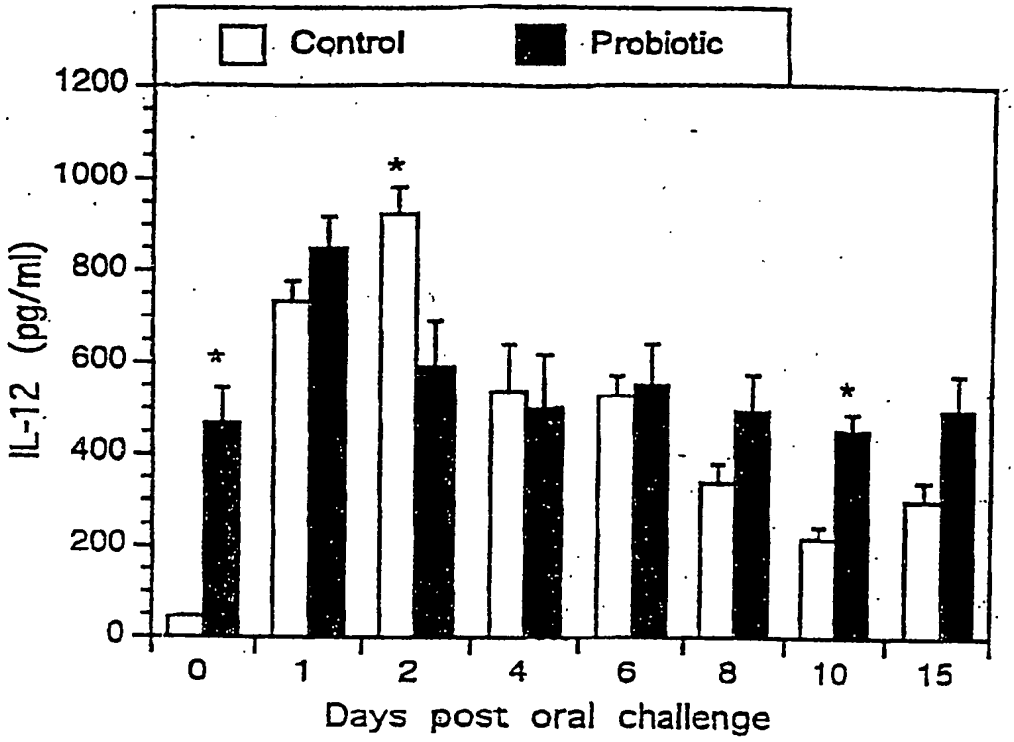

Control
Probiotic
1200
1000
800
600
400
200
0
IL-12 (pg/ml)
*
*
*
0
1
2
4
6
8
10
15
Days post oral challenge

Figure 3

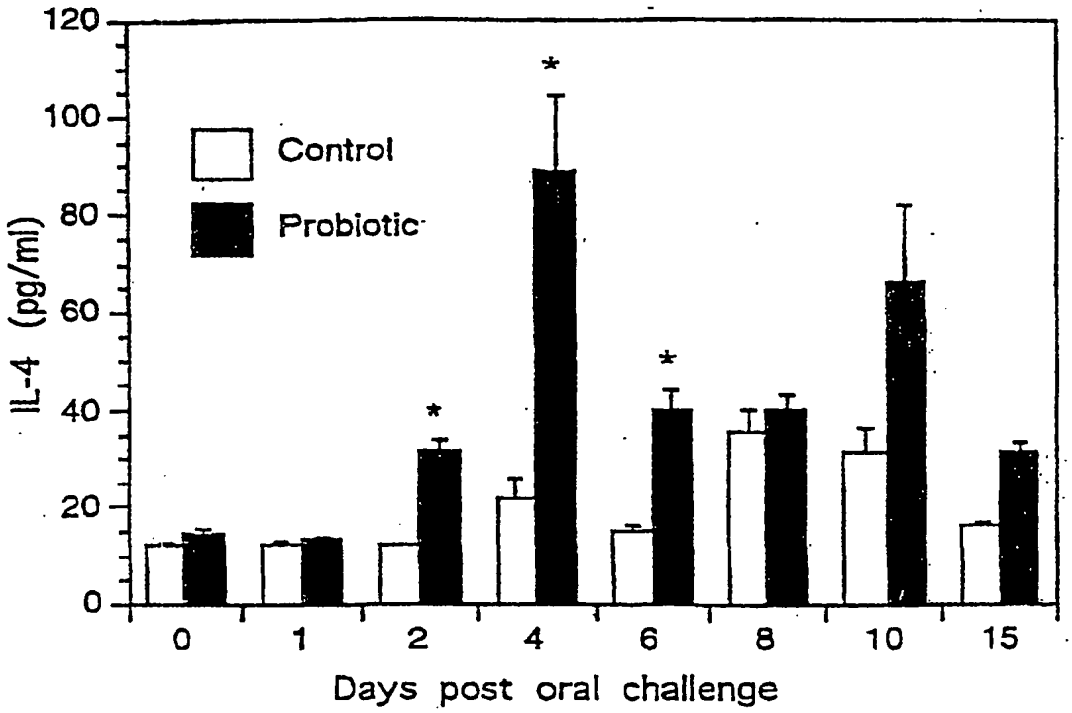
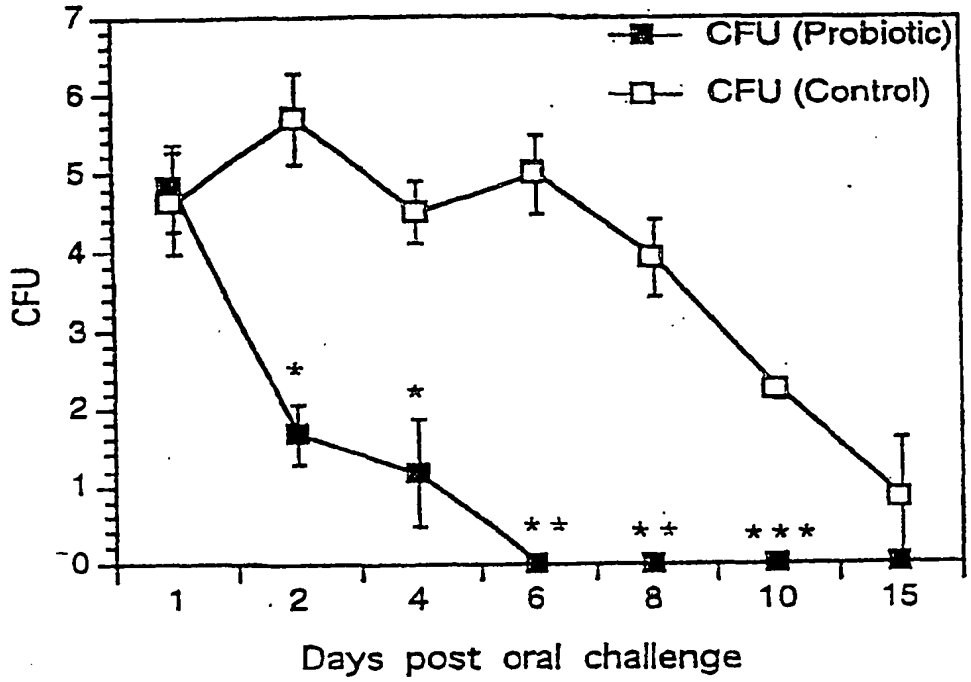

120
100
80
60
40
20
0
IL-4 (pg/ml)
Control
Probiotic
*
*
*
0
1
2
4
6
8
10
15
Days post oral challenge

Figure 4



* P < 0.05, ** P < 0.01, *** P < 0.001

Figure 5

Saliva IFN-γ

Saliva IFN-γ

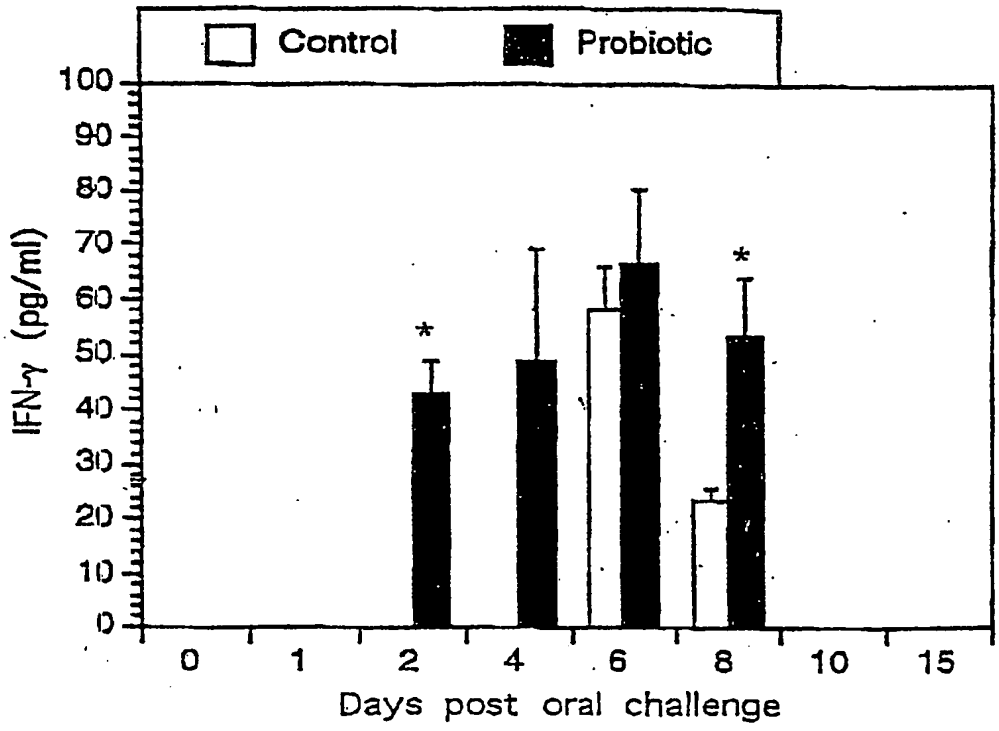


Figure 6

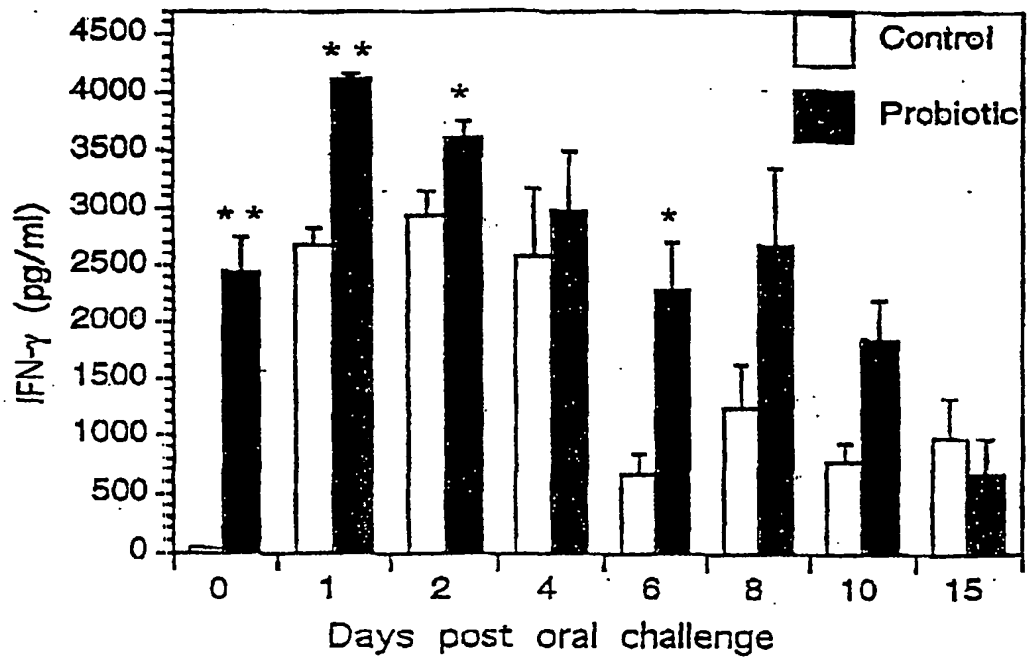
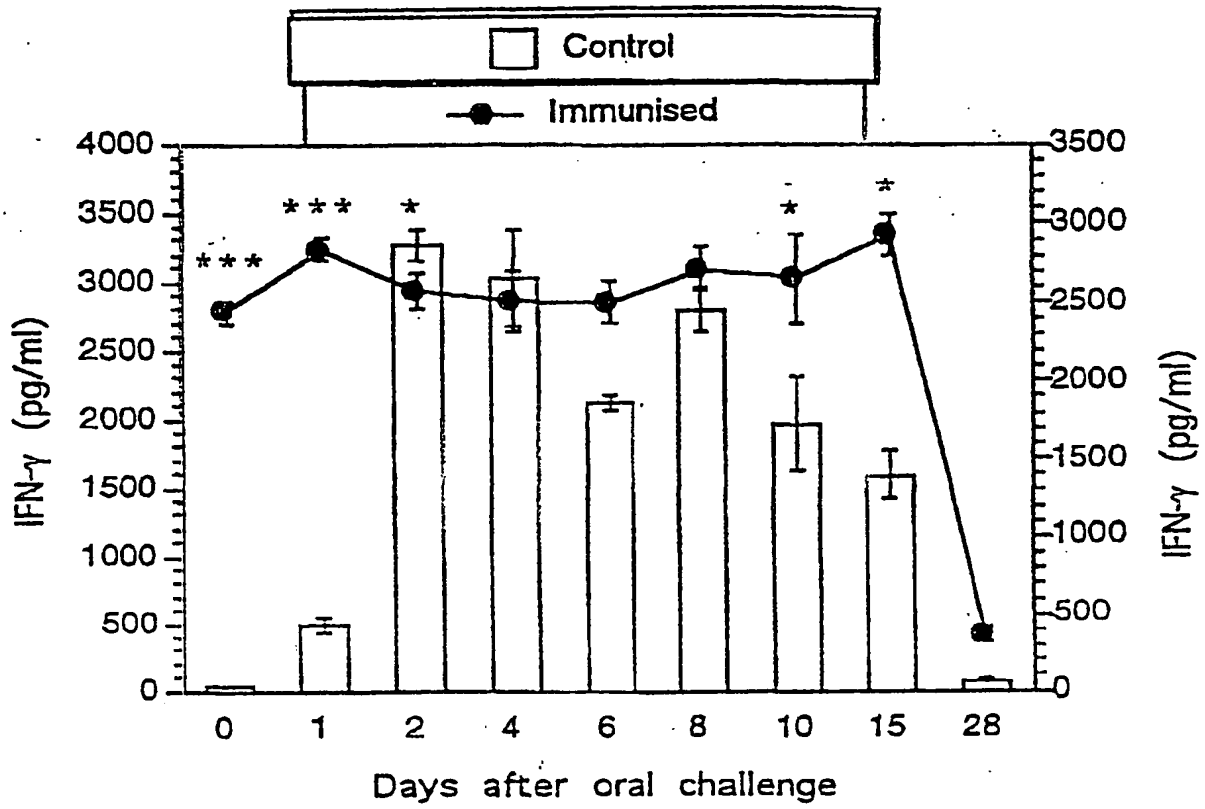
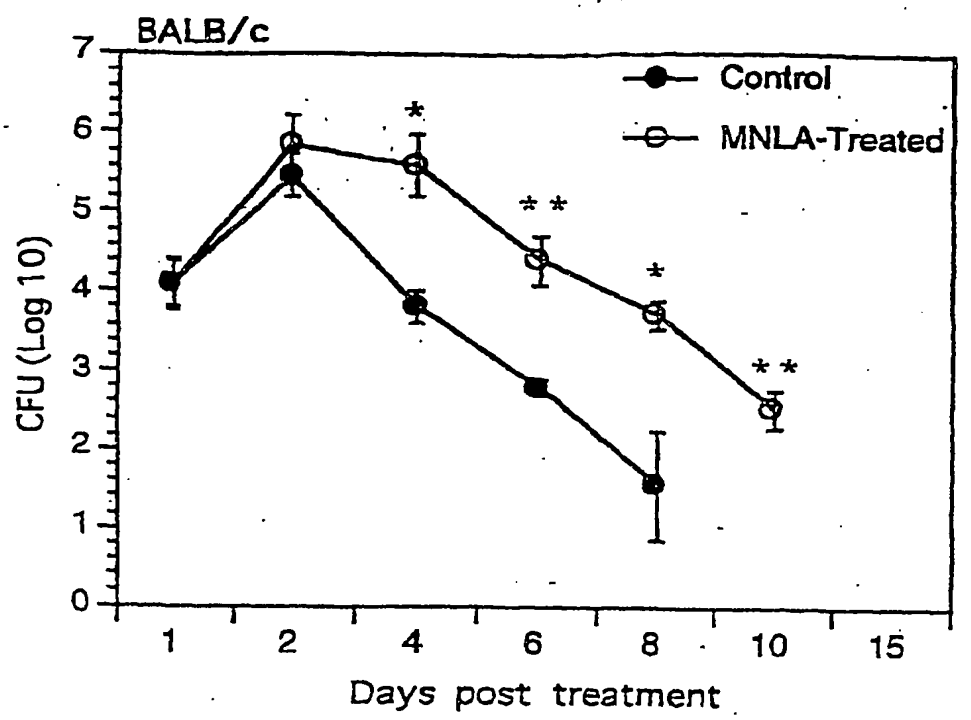

4500
4000
3500
3000
2500
2000
1500
1000
500
0
IFN-γ (pg/ml)
* *
* *
*
*
Control
Probiotic
0
1
2
4
6
8
10
15
Days post oral challenge

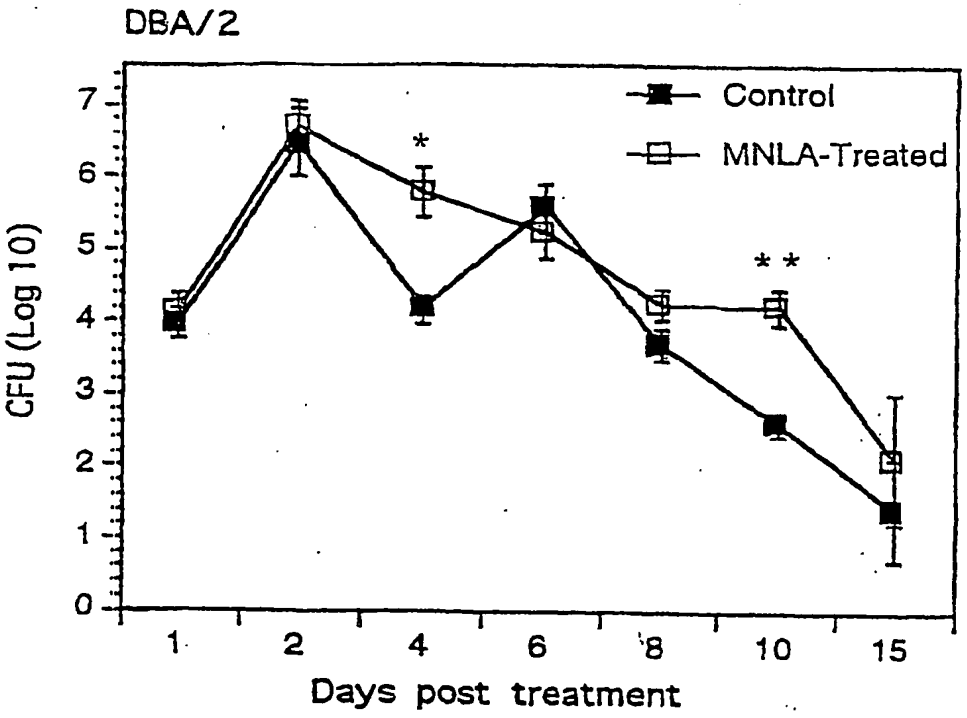
Figure 7



* P < 0.05, *** P < 0.0001

Figure 8





* P < 0.05, ** P < 0.01

Figure 9

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU01/00726

A. CLASSIFICATION OF SUBJECT MATTER

Int. Cl. [7]: A61K 35/74, 39/02, 39/04, 39/07, A61P 31/04, 1/00, 1/04, 1/14, 11/00, 15/02

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
A61K with keywords as below

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
AU: IPC as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
WPAT: Probiotic, lactobacillus(w)acidophilus, casei, mycobacterium vaccae and A61K

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 99/17788 A (Abbott Laboratories) 15 April 1999 Whole document, especially pages 1-12	1-50
X	WO 99/07393 A (Teodorescu) 18 February 1999 pages 1-5 and 22	38-41, 44,45
X	WO 98/55131 A (Essum AB) 10 December 1998 Whole document	6-11, 17-23, 29-33, 38-45,

[X] Further documents are listed in the continuation of Box C [X] See patent family annex

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search
23 July 2001

Date of mailing of the international search report
26 July 2001

Name and mailing address of the ISA/AU

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU01/00726

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 98/26790 A (Stanford Rook Ltd.) 25 June 1998 Entire document	7-9, 14, 17 -23, 29-50
X	WO 97/36603 A (Commonwealth Scientific And Industrial Research Organisation) 9 October 1997 Entire document	1-23, 27-33, 38-50
X	WO 98/23727 A (Bio K + International Inc.) 4 June 1998 Entire document, especially pages 1-12, 18-20	1-23, 27-33, 38-50
X	US 4591499 A (Linn, L.L. et al) 27 May 1986 Columns 1-8	1-3, 7-11, 15-23, 27-33, 38-46
X	EP 353581 A (Dr. A. Tosi Farmaceutici S.r.l.) 7 February 1990 Pages 1-6	7-11, 17-23, 27-33, 38-46,
X	EP 199535 A (The New England Medical Centre Hospitals Inc.) 29 October 1986 Pages 1-20	6-11, 17-23, 27, 29-33, 38-49

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.
PCT/AU01/00726

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report		Patent Family Member					
WO	9917788	NONE					
WO	9907393	AU	87540/98				
WO	9855131	AU	80460/98	EP	1005353	NO	995794
		SE	9702083				
WO	9826790	AU	77355/98	BR	9713959	CN	1246056
		EP	951289	FI	991384	NO	992957
		HU	200001602				
WO	9736603	AU	21451/97				
WO	9823727	AU	51137/98	EP	951531		
US	4591499	NONE					
EP	353581	IT	1227154	JP	2174675	US	5176911
		ZA	8905936				
EP	199535	AU	56112/86	DK	1701/86	FI	860194
		FI	923829	JP	61280433	NO	861483
		US	4839281	US	5032399		

END OF ANNEX

Lactic Acid Bacteria and their Effect on the Immune System

Current Issues in Intestinal Microbiology 2001. 2(1): 27-42.

Lactic Acid Bacteria and their Effect on the Immune System

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[2]Instituto de Microbiología, Facultad de Bioquímica, Química y Farmacia, Universidad Nacional de Tucumán, Argentina

[3]59 Ryeish Green, Three Mile Cross, Reading RG7 1ES, UK

Abstract

Lactic acid bacteria (LAB) are present in the intestine of most animals. The beneficial role played by these microorganisms in the humans and other animals, including the effect on the immune system, has been extensively reported. They are present in many foods and are frequently used as probiotics to improve some biological functions in the host. The activation of the systemic and secretory immune response by LAB requires many complex interactions among the different constituents of the intestinal ecosystem (microflora, epithelial cells and immune cells). Through different mechanisms they send signals to activate immune cells. Thus the knowledge of the normal intestinal microflora, the contribution of LAB and their role in the numerous functions in the digestive tract as well as the functioning of the mucosal immune system form the basis for the study and selection of a probiotic strain with immunostimulatory properties. In the selection of LAB by their immunostimulatory capacity it helps to know not only the effect which they have on the mucosal immune system, but the specific use to which these oral vaccine vectors are being put. Although there are reports of the protection of animals and humans against diseases such as microbial infections and cancer, more work remains to be done on the factors affecting the design of oral vaccine vectors and the use of LAB for therapeutic purposes. The basic knowledge of LAB

Immunostimulation and the criteria for selection of LAB by their immunostimulatory capacity, will be extensively discussed and appraised in this review.

Introduction

All warm-blooded vertebrates live in symbiotic association with a complex population of microorganisms which inhabits their gastrointestinal tract. One of the benefits which the host animal derives from this relationship is an enhanced resistance to infectious diseases (Fuller, 1992, 1997). Thus conventional animals with a complete gut microflora are more resistant to infection than are germfree animals. The detailed basis for this difference is not known but it seems certain that changes in immunity are likely to be involved. The gut microflora stimulates mainly a local response at the gut wall. This mucosal immunity is an important element of the animal's immune status because it is responsible for the control of infections as well as inducing tolerance to environmental and dietary antigens.

Under natural conditions the level of immunity is adequate, but under domesticated conditions, stress factors cause deficiences to occur which render the animal vulnerable to infection. Under these circunstances, supplementation with live microorganisms to repair the deficiences in the composition of the gut microflora can stimulate an immune response and restore the animal's resistance to infection. These supplements known as probiotics have been defined as: "live microbial food supplement which beneficially affects the host animal by improving its intestinal microbial balance" (Fuller 1989).

This definition includes not only preparations specifically designed to act as probiotics but also the traditional yogurts and other fermented products where the benefits confered on the consumer may be incidental to their primary role as a tasty and nutritious food. By far the most commonly used microorganisms in probiotic products are the lactic acid bacteria (LAB) and it is important to know how these LAB affect the immune status of the consumer.

The probiotic approach is attractive because it is a reconstitution of the natural condition; it is a means of repairing a deficiency rather than the addition of foreign chemicals to the body which may have toxic consequences or, as in the case of antibiotics induce resistance and compromise subsequent therapy.

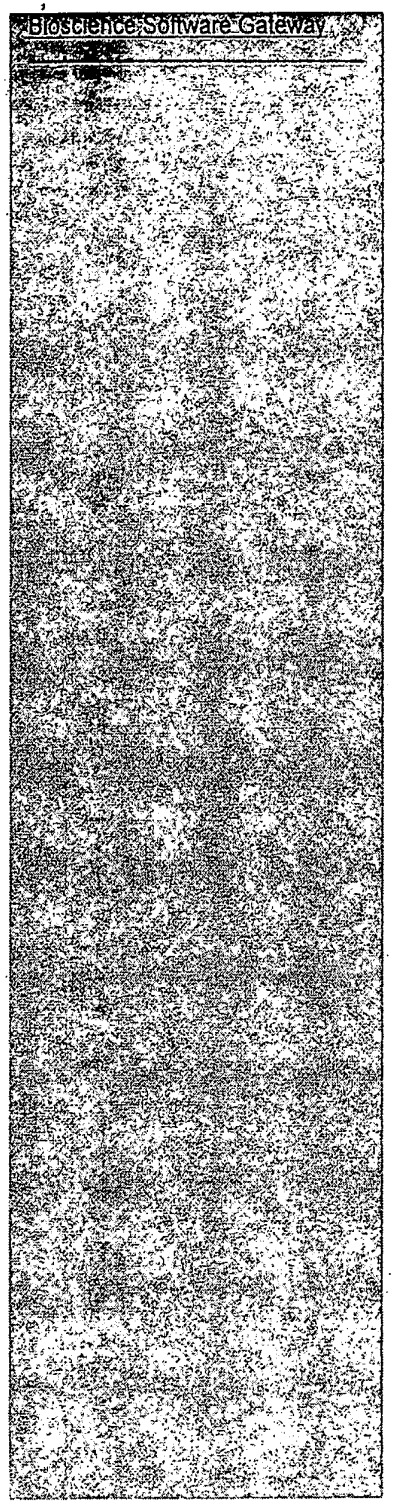
The discovery that probiotics can stimulate an immune response (Fuller and Perdigón, 2000) provides a scientific basis for some of the observed probiotic effects. This is an important function of probiotic preparations and a rapidly developing area of research. The scientific results and their practical implications will be reviewed and discussed in the remainer of this review.

The Role of Mucosal Immunity

The intestine is the largest immunological organ in the body. It contains 70-80% of all the IgA producing cells which exceeds the total production of all other immunoglobulin classes in the body.

The most important factor for local immunity is the cellular migration of specific activated B and T cells from the Peyer's patches (PP) which are the inductive sites to the distant mucosal sites such as respiratory, genitourinary tract and various secretory glands. They also return to the intestinal lamina propria and epithelium (Phillips-Quagliata and Lamm, 1988, Weisz-Carrington *et al.*, 1979). The specific migration of these immune cells is the basis of the Common Mucosal Immune System (Scicchitano *et al.*, 1988, Mestecky *et al.*, 1994).

The structure and function of the intestinal mucosa are designed to supply different biologically active molecules such as gastrointestinal peptides, enteroglucan, trefoil peptides, hormones, prostaglandins, growth factors (Levis *et al.*, 1992, Wright, 1995, Jankowsky *et al.*, 1994), and mucus produced for the goblet cells.



External secretions such as tears, saliva, milk, intestinal, genital and bronchial fluids have non-specific antiviral, antibacterial and antiparasitic activity due to inorganic and organic acid, lysozyme, lactoferrin, peroxidases and interferons (Mc Ghee and Kiyono 1992, Brandtzaeg, 1989, Kilian *et al.,* 1988, Mestecky *et al.,* 1988, Strobel, 1995). Intestinal peristalsis is another important mucosal mechanism of defense for the host preventing the colonization of the gut by foreign microorganisms ingested with the food. The intestinal epithelium is also a barrier for the antigens present in the daily diet. This barrier is maintained by the tight junction at the apical site of the epithelial cell, by phospolipids and proteins that cover the microvilli of these cells.The lamina propia of the intestine is rich in immune cells such as lymphocytes, plasma cells, neutrophils, eosinophils, macrophages and mast cells. The lymphocytes are associated not only with the lamina propria but also with the epithelium: intraepithelial lymphocytes (IEL). They are active in maintaining the hyporesponsiveness at the intestinal level.

Lymphoid nodes are present in the small and large intestine. In the small intestine they constitute the Peyer's patches where microvilli are absent and mucus production is reduced.

When an antigen is orally administered the main immune response induced is in the form of hyporesponsiveness. It is known as oral tolerance and avoids an increase in the inflammatory immune response that can lead to an enhancement in intestinal permeability.

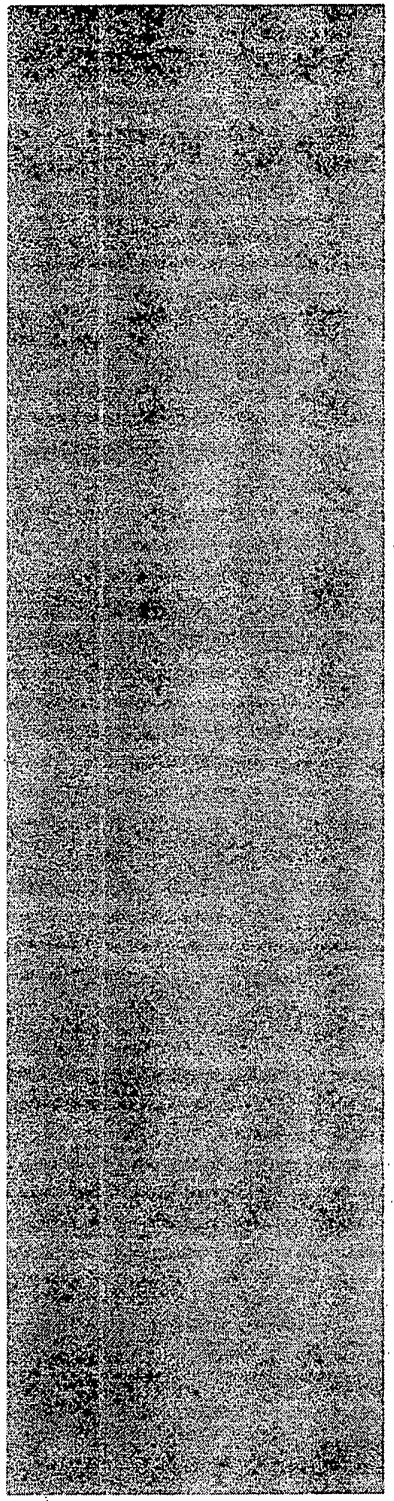
In general, soluble antigens give a strong oral tolerance. Particulate antigens, especially bacteria or virus, favour the induction of the immune response. The hyporesponsiveness is related to the dose of the antigen administered. Lower or higher doses of antigens can induce oral tolerance through supression of cytokines such as interleukin 10 (IL10) or transforming growth factor b (TGFb) or by clonal deletion (Elson and Zivny, 1996)). However, oral tolerance can be abrogated and an immune response induced.

The entry of the antigens by the oral route is essential to induce a mucosal immune response. This fact was determined in germfree mice receiving a diet free of antigen where it was demonstrated that the presence of a microflora increased the number of IgA secreting cells in the lamina propria of the intestine, mesenteric node or in the bone marrow (Hoojkaas *et al.,* 1984, Bos *et al.,* 1987). In similar germfree studies with antigen free diet the levels of the IgM, IgG and IgA in the serum were also diminished (Wostmann and Pleasants, 1991). They concluded that the level of IgA is mainly dependent on the presence of microflora, whereas for IgG the diet is the more important factor. It would seem that the IgM is not influenced by environmental antigens.

The gut associated lymphoid tissue (GALT) is characterized by the development of a systemic hyporesponsiveness or oral tolerance. This suppressor immune response avoids immunological reactions induced by chronic stimulation by the microbial and other antigens contained in the diet. Oral tolerance is mainly induced by antigens of T dependent cells; the presence of $CD8^+$ T cells is required in this process (Challacombe and Tomasi, 1980, Mowatt, 1987, Mattingly and Waksman, 1978). The lipopolysacharide (LPS) originating in the normal enteric microflora is involved in the maturation of the T cell precursors of the T suppressor cells responsible for oral tolerance at the gut level. This was demonstrated in germfree mice that are unable to induce oral tolerance, but this can be reconstituted by the intestinal colonization with the enteric microflora or by LPS ingestion (Kiyono *et al.,* 1982, Michalek *et al.,* 1982, Michalek *et al.,* 1983). It is also known that the maturation of lymphocytic function is controlled by the normal microflora and by the non-colonizing, non-pathogenic exogenous bacteria that pass through the gastrointestinal tract. The maintainence of a constant number of IgA secreting cells in the intestine, in normal conditions, has been atributed to the Gram negative microflora specially *Bacteroides* spp. (Porter and Allen, 1989) It was also demonstrated that the muramyl dipeptide (MDP) of the Gram positive cell wall activates immune cells such as macrophages, and B and T lymphocytes (Lise and Audibert, 1989) asssociated with the intestinal mucosa.

Induction of Mucosal Immunity

To achieve an effective oral immune response the participation of almost all of the immune cells associated with the gut is necessary. Macrophages, regulatory T cells and effector B and T lymphocytes induce the protective IgA associated with the mucosal surfaces. This process can be divided into inductive sites where antigen is encountered and initial responses are induced, and effector sites where IgA plasma cells are found and where the production of s-IgA antibodies results in local immune protection. Although physically



separated they are functionally interconnected (Mestecky and Mc Ghee, 1992, Czerkinsky *et al.,* 1993).

The IgA inductive sites have been extensively studied in GALT, which is represented by the Peyer's patches (PP), the appendix and the small lymphoid nodules in the large intestine. The bronchus associated lymphoid tissue (BALT) (Bienestock and Clancy, 1994) shares many similarities with GALT and it is also probable that lymphoid nodules are present in the urogenital tract.

The PP contains a "dome" region enriched by lymphocytes, macrophages and some plasma cells. This area is covered by a unique epithelium that contains specialized cells termed microfold (M) cells, which have short microvilli, small amount of cytoplasm and few lysosomes. Its function is the uptake and transport of lumenal antigens and small parasites. The antigen uptake by M cells does not result in degradation, but delivers intact antigens into the underlying lymphoid tissue (Mc Ghee and Kiyono, 1992, Neutra and Krahenbuhl, 1996).

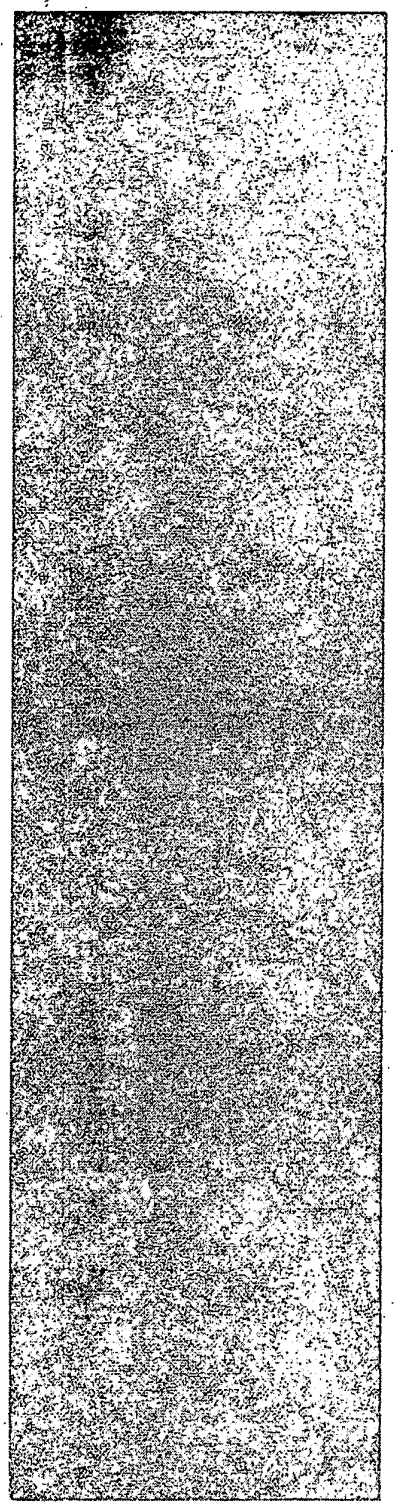
Peyer's patches are considered germinal centres where B cells change IgM to IgA and affinity maturation occurs. In this center the majority of the cells are B cells producing IgA. The switch of IgM to IgA is induced by T helper ($CD4^+$) cells of type Th2. The 60% of T lymphocytes present in PP are $CD3^+$, $CD4^+$, $CD8^-$ with properties of T helper and the T cell receptor (TCR) in a/b form, the type $CD8^+$ T cells are also present in PP. The difference between Th1 and Th2 populations with the same $CD4^+$ phenotype is in the cytokines produced. Th1 cells release Interleukin (IL) 2, IL3 and interferon g (IFNg). The Th2 cells produce IL4, IL5, IL6, IL10 (Mosmann and Coffman, 1987). The accesory cells such as dendritic cells and macrophages present in the IgA inductive sites are the antigen presenting cells (APC) and they are engaged in regulation of humoral and cellular immune responses for mucosal protection.

Following antigen stimulation in PP and its presentation to B and T cells, the antigen induced B and T cells ($CD4^+$ and $CD8^+$) are able to migrate *via* efferent lymphatics and through the mesenteric node; they reach the systemic circulation through the thoracic duct and repopulate not only the lamina propria of the intestine but other distant mucosal sites such as respiratory, urogenital, mammary and salivary glands. The result of this process is that by oral stimulation, distant mucosal sites can be repopulated with IgA producing cells to protect these surfaces. This phenomenon has been termed the Common Mucosal System (Cebra *et al.,* 1991). However, in spite of the scientific evidence of the ability of the common mucosal system to induce a good local mucosal response, local stimulation is also required.

In the process of antigen uptake by M cells the antigen must associate with the M cells in the apical membrane of PP which have abundant glucoconjugates. These lectin binding sites coat the antigens. Most of the bacteria have adhesin on their surface that permits the adhesion to the M cells and their subsequent contact with the immune cells of the PP (Bye *et al.,* 1984, Clark *et al.,* 1993, Falk *et al.,* 1994) stimulating a mucosal immune response.

Although in the past it has been suggested that PP is the only site of mucosal immune response induction, recent studies have demonstrated that the epithelial intestinal cells are another important component of the mucosal immune system. These cells were extensively studied for their essential role in the secretory and absorptive processes (Kagnoff, 1996). However, many studies showed that the intestinal epithelial cells can be regarded as non-professional antigen presenting cells. Epithelial cell express histocompatibility antigen (HLA) class I and II molecules (Mayer *et al.,* 1991, Blumberg *et al.,* 1991) and they can release interleukins such as IL6 and IL8 (Mulder *et al.,* 1990, Reinecker and Pololsky, 1995). The epithelial cells communicate with other mucosal cells *via* a spectrum of mediators that act on the intestinal epithelial cells as well as on the intraepithelial lymphocytes, lymphoid cells, mononuclear phagocytes, neutrophils, mast cells and eosinophils present in the lamina propria. The cascade of mediators is regulated both to induce or to down-regulate appropiate host immune and inflammatory responses at mucosal surfaces.

If the antigen interacts with the epithelial cells, it can be partially taken up degraded and presented to the immune cells. Another possibility is that undigested particles are eliminated through the intercellular space by the portal circulation and carried to the liver where they are cleared (Walker and Sanderson, 1995).



Whatever the route of the secretory immune response induction, the main immune response is the humoral immune response by IgA^+ cells and secretory IgA production. This immunoglobulin is present in its two subclasses IgA1 and IgA2 and they have an important role in the protection of mucosal surfaces against pathogens. Secretory IgA (s-IgA) is a dimeric molecule bound by the join chain "J" produced by the plasma cells and in the secretions it has a small glycoprotein of 80 Kd called the 'secretory component' which in responsible for resistance to gastric juice, fatty acids and bile acids. This secretory component is synthetized by the epithelial cells and is combined also with IgM antibodies (Brandtzaeg, 1995).

When IgA is secreted from plasma cells in the lamina propria, it can enter enterocytes by receptor-mediated endocytosis and the IgA is transported in vesicles through the cell into the luminal secretions, where it has the oportunity to complex antigens. IgA can also act at the intraepithelial level neutralizing viruses that are infecting the cells (Mazanec *et al.,* 1992, Mazanec *et al.,* 1995) and in the lamina propria it can bind antigens. This complexed antibody is internalized and transported to the lumen in a manner similar to that of free IgA. This is an excretory function of IgA (Lamm *et al.,* 1996). In the mucosal immune response the release of cytokines by the immune cells associated with the mucosal and intestinal epithelial cells play an important role in triggering the immune response or in stimulating the inflammatory immune response. As the profile of cytokines is different in different immune cells, alteration of the ratio of one type such as $CD4^+$or $CD8^+$ in the number of these immune cells present in the lamina propria can lead to an increase in the inflammatory immune response. It has been demonstrated that tumour necrosis factor (TNF), IFNg and IL4 have a modulatory effect on the secretory component expression (Phillips *et al.,* 1990).

The cytokines released by Th2 cells are involved in the induction of the IgA immune response. The IL6 and IL8 released by the epithelial cells are proinflammatory, so an intense epithelial stimulation can favour an inflammatory immune response.

Although it has been shown that the pattern of cytokines produced by T helper cells associated with the lamina propria is the same as that of those produced in the PP, they are functionality less active (Williams *et al.,* 1997).

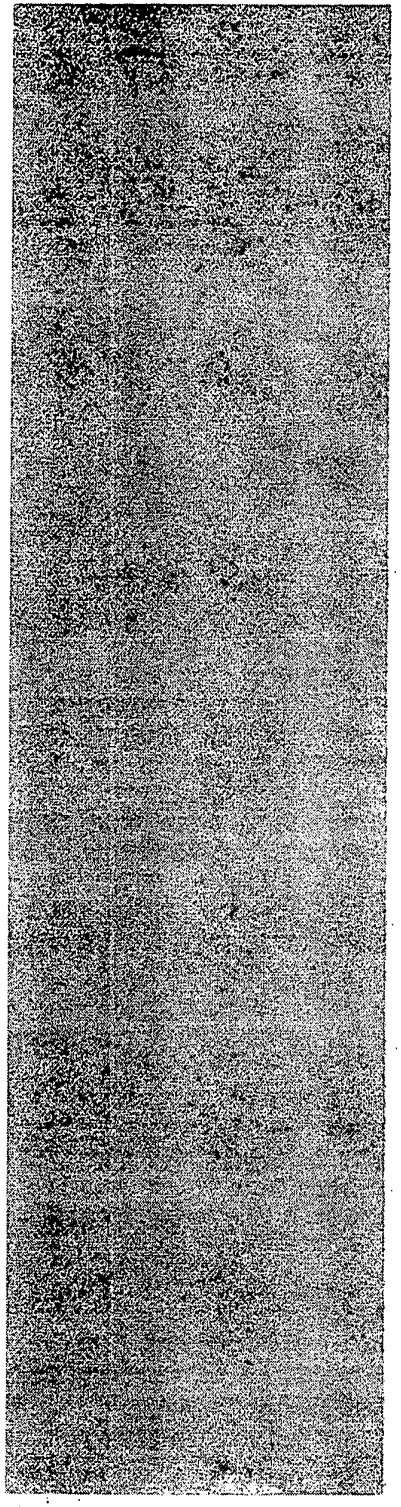
IgA synthesis involves a complex network of signals between antigen, immune cells and cytokines. Weiner (1997) proposed three ways of interaction with the intestinal cells to evoke an immune response: 1) through M cell from PP, 2) through the epithelial cell with processing and presentation or not of the antigen 3) interaction with the epithelial cells and elimination of the antigen by portal circulation or by inducing a local immune response activated by the release of cytokines.

To enhance mucosal immunity a number of different compounds have been found to have adjuvant properties when given orally together with antigen. These antigen delivery systems, designed to stimulate the mucosal immune response, have been studied almost exclusively in experimental animals (Michalek *et al.,* 1994). It has been proposed that such systems include incorporation of antigens into particles (liposomes, proteasomes, immunostimulatory complexes, ISCOM, chemical or biological linkage of antigens to cholera toxin (CT) or fraction b (CTb), expression of antigens in recombined viruses, (poliovirus, adenovirus and vaccinia) bacteria (*Salmonella*, BCG and lactobacilli) or plants (potatoes, tomatoes, spinach) or mucosal immunization with DNA.

However, the efficiency in humans of the mucosal antigen delivery system is rather limited. Only liposomes, microspheres, recombinant *Salmonella* and adenovirus have been used in human trials (Mestecky *et al.,* 1997). The other mucosal antigen delivery systems mentioned await evaluation in human vaccines.

Liposomes and biodegradable microspheres have been used for systemic and mucosal immunization of animals (Kersten and Crommelin, 1995, Childers *et al.,* 1994, Mestecky *et al.,* 1994, Tacket *et al.,* 1992, Lise and Audibert, 1989). Their use in humans is limited to enteral immunization.

Cholera toxin (CT) is a potent mucosal immunogen and enteric adjuvant and its non-toxic b subunit (CTb) in pure or recombinant form has been approved for use in humans (Jertborn *et al.,* 1992). It enhances the secretory IgA antibody response to the coupled antigen, as well as circulating IgG antibody.



The generation of the mucosal immune response through the common mucosal immune system as well as th ir compartments has led to the use of individual vectors by virtue of their ability to colonize or infect selected inductive sites such a the upper respiratory tract mucosa, the gut or the female genital mucosa.

Live vectors by themselves are better antigens than the inserted gene product from other microorganisms. H vever, they can induce a greater immune response against the vector that may lead to its elimination restricting the use of the live-ve tor based vaccines.

In vivo assessment of mucosal immune response is limited if the studies can only be performed in animals o human beings. Evaluation of the effect of the different adjuvants on the local and generalized mucosal immune response co be assessed by many *in vitro* techniques.

Effect of LAB on the Immune Response

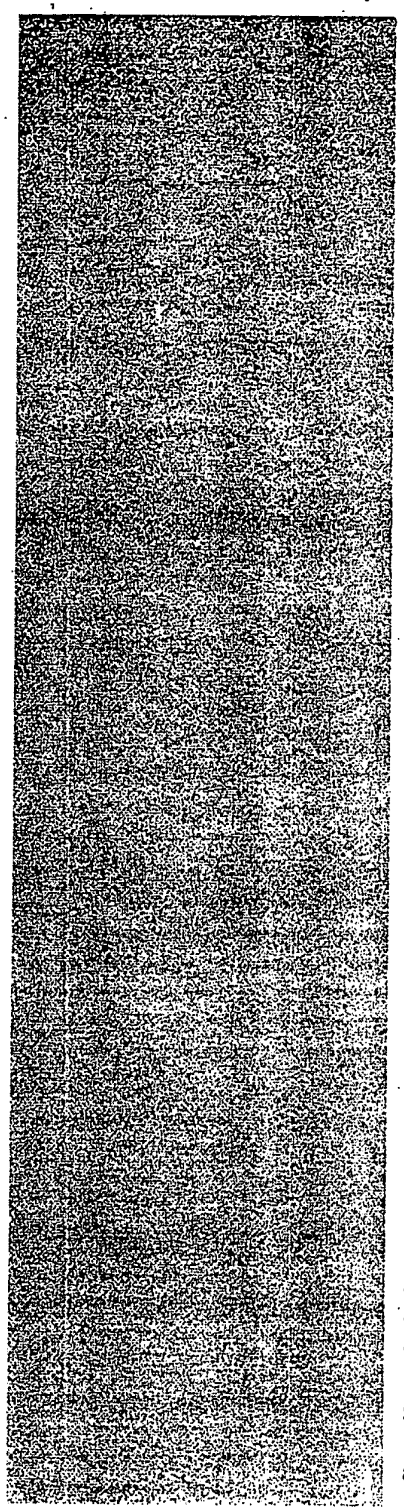
Enhancement of Systemic Immune Response

The role played by lactic acid bacteria in various biological functions of the host has been extensively reporte Numerous studies have demonstrated that LAB and milk fermented with LAB had antitumour activity and that they were able to event intestinal infection; these observations imply an active participation of the immune system.

Early studies (Bloksma *et al.*, 1979) demonstrated that viable *Lactobacillus plantarum* administered intraperit eally stimulated the delayed type hypersensitivity (DTH) reaction, whereas non-viable bacteria acted as adjuvants for antibody pr luction. Kato *et al.* (1983) demonstrated that *Lactobacillus casei* inoculated intraperitoneally activated peritoneal macrophages, i creasing their phagocytic capacity. They also observed an enhanced activity of the mononuclear phagocytic system measu d by an increase in the colloidal carbon clearence index rate. This would mean that *L. casei* has an immunopotentiator effect. Saito *al.* (1983) demonstrated that subcutaneous administration of *L. casei* induced an increase in the production of circulatin antibodies against *Pseudomonas aeruginosa* and sheep red blood cells (SRBC). The intravenous or intraperitoneal inoculation *L. casei* induced the activation of natural killer cells (NK) which play an important role in preventing tumour development (Kato *et a* , 1984). The strain of *L. casei* assayed in this study proved to be as effective as other microorganisms used as immunopotentiators. H wever, unlike *Propionibacterium acnes* (previously *Corynebacterium parvum)* or *Mycobacterium bovis*, *Bacillus Calmette-G erin* (BCG), it did not produce hepatomegaly or splenomegaly which are very common side effects of these immunomodulators (Y utake *et al.*, 1984b).

Hashimoto *et al.* (1985) demonstrated by *in vitro* assay that Kupffer cells and the immune cells associated wi spleen or lung and peritoneal macrophage were activated by *L. casei* administration. De Simone *et al.* (1986) observed that the y gurt given to humans induced production of the cytokine interferon g (IFN g) when their circulating blood lymphocytes were stimula d with concavalin-A. In spite of these beneficial properties described for the LAB, there are other reports indicating that LAB do not a vays produce beneficial effects on the host. Sharpe *et al.* (1973), observed that *Lactobacillus rhamnosus* was associated with endoca litis or abscesses. Some strains of *Lactobacillus acidophilus* and *L. plantarum* may possess undesirable properties. It was also d monstrated (Iwasaki *et al.*, 1983) that *Lactobacillus arabinosus* administration to mice bearing chemically induced intestinal tumour c uld enhance the tumour effect of the carcinogen.

Although these previous studies encouraged the use of certain lactobacilli as immunopotentiators for therape tic purposes, there is still much to be discovered. For example a) it is important to know the type of immune cells that the LAB are a le to stimulate, to know if the immune response induced will be beneficial or not for the host (inflammatory or specific immune respon e) b) which is the most active strain, c) the dose required for maximum effect, d) when it should be administered and e) is it safe to u e LAB or fermented milks in an immunosuppressed host?. We must also be reassured that substances or bacteria used as immu omodulators do not have harmful effects on the host, for example can we be certain that side effects will not occur as a result of l g term administration.



Since lactic acid bacteria are usually ingested with the daily diet it is also important to know the mechanisms of action of these bacteria not only on the systemic immune system but, on the mucosal immune system. All of these parameters mentioned before must be taken into account for the optimal induction of the mucosal immune response.

In previous studies in our laboratory, using mice as the experimental model, we demonstrated that the oral administration of *L. casei* CRL 431, *L. acidophilus* CRL 924, *Lactobacillus delbrueckii* subsp. *bulgaricus* CRL 423 and *Streptococcus thermophilus* CRL 412 were able to increase the non-specific immune response measured by the phagocytic activity of peritoneal macrophages and by the release of lysosomal enzymes such as b glucuronidase and b galactosidase (Perdigón *et al.*, 1986a). We also demonstrated that LAB increased the phagocytic activity of the mononuclear phagocytic system measured by the colloidal carbon clearence test (Perdigón *et al.*, 1986b). The effect observed on the non-specific immune response when the LAB were administered by the oral route was comparable to those obtained when the LAB were injected intraperitoneally.

When we analyzed the influence of the oral administration of the LAB under study on the specific systemic immune response, we found that the LAB stimulation induced an increase in the IgM levels against sheep red blood cells (SRBC) measured by the plaque forming cells test (PFC) (Perdigón *et al.*, 1986c, Perdigón *et al.*, 1987, Perdigón and Alvarez, 1992). We also analyzed the effect of a mixture of *L. acidophilus* and *L. casei* in a fermented milk and conventional yogurt (mixture of *L. debrueckii* ssp. *bulgaricus* and *S. thermophilus*) on the systemic immune response. We found that the mixture of *L. acidophilus* and *L. casei*, microorganisms were better able to survive in the intestinal tract and were more effective than yogurt (Perdigón *et al.*, 1988, Perdigón *et al.*, 1989, Perdigón, Alvarez and Medici, 1992). We also investigated whether or not the long term administration of LAB induced side effects. We observed that after treatment for 7 consecutive days at a dose of 10^9 cell/day/mouse the bacteria did not induce hepato- or splenomegaly.

Antitumour Activity

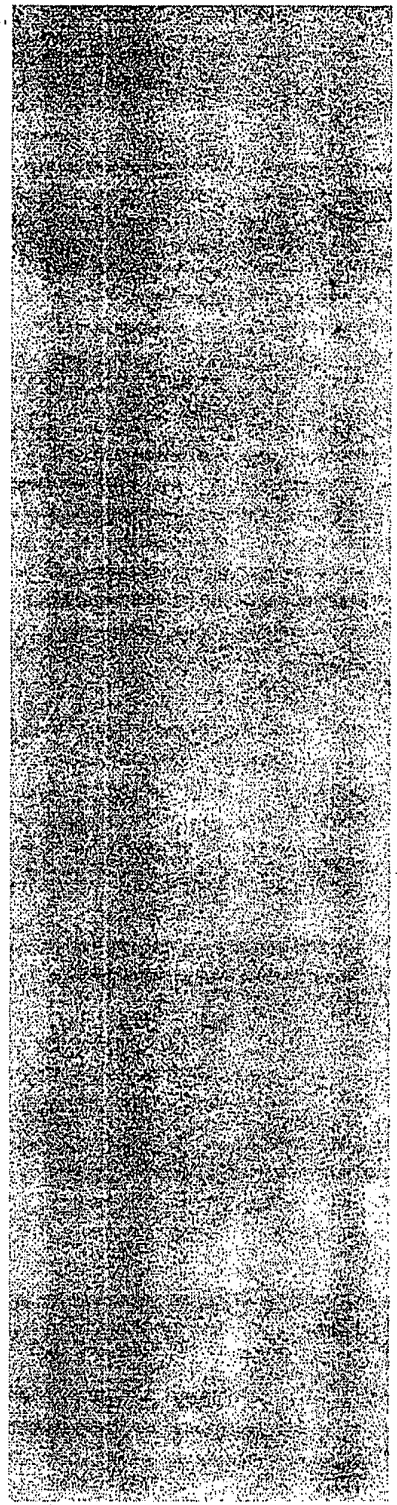
During the last two decades, the anticarcinogenic properties of LAB and of yogurt have been extensively studied. A great emphasis has been laid on the antitumour property exerted by yogurt. Most of the scientific evidence came from animals models but no evidence has been reported from humans.

Shahani *et al.* (1983) demonstrated in mice fed with fermented colostrum that the growth of an experimental tumour was inhibited. Reddy *et al.*(1983) and Ayebo *et al.* (1982) studied whether the antitumour effect observed was exerted by the presence of LAB in the fermented milks or by components of their cell wall, or by products produced as a consequence of the fermentation process. Ayebo (1981) isolated a dialysable antitumour component from yogurt. Goldin and Gorbach (1980) showed in mice that *L. acidophilus* orally administered induced a decrease in the incidence of the colon cancer caused by 1-2 dimethylhydrazine dihydrochloride (DMH).

Kato *et al.* (1981, 1985) found that the intraperitoneal administration of *L. casei* inhibited tumour growth in syngeneic and allogenic mice, and that the effect was dose dependent. Yasutake *et al.* (1984a) observed that the intratumour administration of *L.casei* produced a total inhibition of the tumour, while simultaneous injection of *L. casei* in different body sites had no effect on tumour growth.

Matsuzaki *et al.* (1985) showed tumour inhibition by intravenous administration of *L. casei* in syngeneic mice and guinea pigs with carcinoma of the lung and liver tumour respectively. Bogdanov *et al.* (1975) and Sekine *et al.* (1985) demonstrated that the antitumour capacity of *L. delbrueckii* subsp. *bulgaricus* or *Bifidobacterium infantis* respectively was related to a fraction of the cell wall (peptidoglycan).

Several papers have reviewed the evidence for the supression of carcinogenesis by LAB (Friend and Shahani, 1982, Fernandes *et al.*, 1987, Hosono, 1988, Gilliland, 1990 a, Adachi, 1992, Ballongue, 1993, Nadathur *et al.*, 1994) based on research concerning antimutagenic and anticarcinogenic activity. There is also evidence in humans that oral supplements of *L. acidophilus* reduce



acitivities of faecal bacterial enzymes such as b- glucuronidase, nitroreductase and azoreductase that are in lved in procarcinogen activation (Gilliland, 1990 b, Hosoda *et al.,* 1992).

Many researchers have studied the biological basis of the antitumour effect of dietary LAB in various anima odels for human cancer. The understanding of the suppression of antitumour activity has led to the conclusion that the LAB could act modulation of the immune response. However, the mechanisms are not the same for different types of tumour. In addition, the peutic antitumour effect by LAB differs as a function of dose, time of administration and the route chosen.

The mechanisms that could be involved are: a) LAB induce an increase in the cytotoxic capacity of macroph es or $CD8^+$ T cells, b) LAB are cytotoxic for tumour cells, c) LAB induce a non-specific local inflammatory reaction inducing a host- diated immunological response against the tumour, d) LAB enhance the cytokines released which are involved with the cellular ap tosis induction, e) LAB give rise to specific immunity to the tumour.

Bifidobacteria are not LAB, but they are frequently included in probiotic preparations and they have shown a tumour activity when administered as preventive or therapeutic agents. These microorganisms are capable of inhibiting tumour gr vth or causing a complete regression of solid tumours (Kohwi *et al.,* 1978, 1982). In the case of *B. infantis,* active component solated from the cell wall were characterized (Tsuyuki *et al.,* 1991, Yasui *et al.,* 1995). This cell wall preparation (whole peptidogli n (WPG) not only exhibited a high capacity to suppress the tumour growth, but also reduced the tumour incidence (Hosono *et* ., 1997). In most of these animal models the antitumour activity of LAB was demonstrated using different routes of administratio but not the oral route. The finding that the oral administration of dairy lactic acid bacteria or fermented milk exerted a therapeutic o ntitumour effect would provide a very attractive form of therapy in humans.

As regards the therapeutic aspect, different authors (Reddy *et al.,* 1983, 1973, Friend *et al.,* 1982, Ayebo *et* , 1981) have demonstrated in mice that feeding with yogurt or yogurt components for 7 consecutive days after intraperiton al inoculation of Ehrlich ascitic tumour cells, produced significant antitumour activity. Asano *et al.* (1986) observed tumour regressio f a carcinoma of the bladder by the daily administration of a fermented milk containing *L. casei.* An antitumour effect of *L. casei* w also observed on a primary colon tumour by oral administration (Kato *et al.,* 1994). In all of these studies viable bacteria were m effective than non-viable ones in producing the antitumour effects.

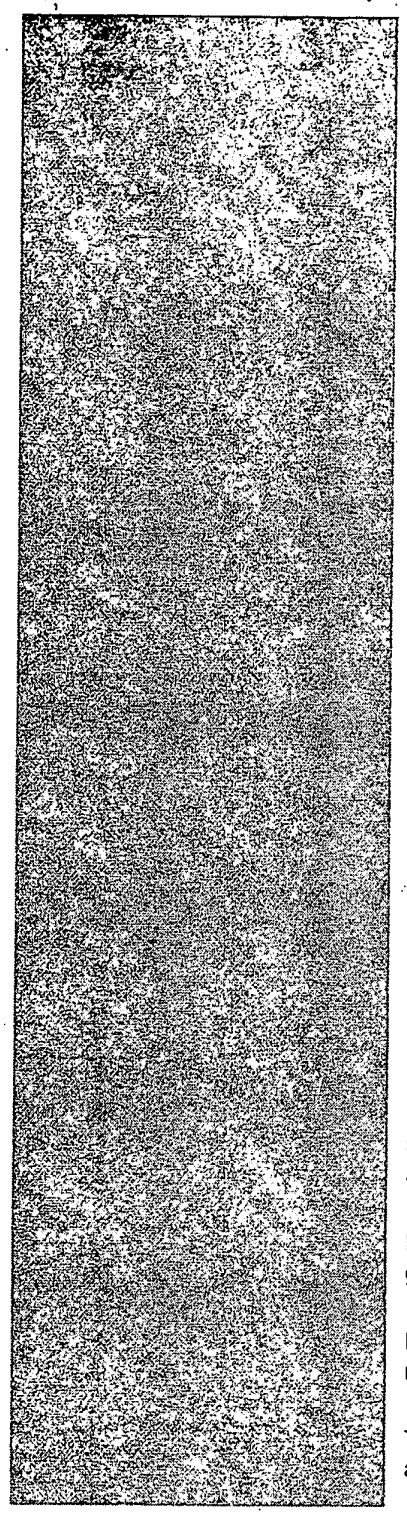
In our laboratory we demonstrated using mice (Perdigón *et al.,* 1993 b, Perdigón *et al.,* 1994, Perdigón *et al.,* 998, Valdez *et al.,* 1997, Perdigón and Oliver, 2000), that oral administration of *L. casei* and yogurt were able to inhibit the tum growth of a chemically induced fibrosarcoma or a carcinoma respectively.

In the inhibition of fibrosarcoma (non-intestinal tumour) by *L. casei,* we observed that the size of the dose wa important, and that *L. casei* was more effective when it was administered as a preventive rather than for therapeutic purposes.

We studied the possible mechanisms involved in this antitumour effect by measuring peritoneal macrophage ctivity, as well as cytotoxic capacity (Perdigón *et al.,* 1995c). We observed an increase in the macrophage activity and also an nhancement of the cytolitic activity of the serum on tumour cells in the animals treated with *L. casei.*

However, we could not demonstrate the exact mechanisms by which *L. casei* inhibited tumour growth in site emote from the immunomodulator penetration route. Perhpas this effect was mediated by cytokines released as a conseque ce of the oral activation of the immune cells associated with the gut.

We also studied (Perdigón *et al.,* 1998) in mice the effect of yogurt on the inhibition of colon tumours induced by DMH. We determined the importance of the dose needed to induce the antitumour effect of yogurt, and found the effect more evide t after 7 or 10 days of feeding. We demonstrated an increase in the IgA^+ B cells and $CD4^+$ T cells present in the large intestine, an a diminution of the CD8



+ T lymphocytes and b- glucuronidase enzyme measured in the intestinal fluid. We also determined an increase in the phagocytic capacity of macrophages infiltrating the tumour (Valdez *et al.*, 1997). We suggest that one of the mechanisms by which yogurt exerts tumour inhibition is through its immunomodulatory activity causing a reduction in the inflammatory immune response. We believe that yogurt also induces the release of different cytokines able to send signals to the proliferative cells inducing apoptosis, with an inhibition of tumour growth.

Effect of LAB Against Intestinal Infections

Lactic acid bacteria and bifidobacteria have their probiotic effects by influencing the biochemical, physiological and antimicrobial activities or changing the composition of the autochtonous or allochthonous intestinal microfloras.

The demostration that LAB are able to inhibit, under *in vitro* conditions, the growth of pathogens including *Salmonella enteritidis* serovar Typhimurium (Gilliland and Speck, 1977) stimulated work on the mechanisms involved in this antibacterial effect. A bacteriocin produced by *L. delbrueckii* subsp. *bulgaricus* has been identified (Pulverer *et al.*, 1993, Nord, 1993) as bulgarican which posseses a wide, *in vitro,* antibacterial activity. In addition, live microbial therapy has been shown in some reports to be more effective than antibiotic administration for treating *Salmonella* infections (Hitchins *et al.*, 1985). This effect may be due to the enhancement of a specific immune response. The protection against *Salmonella* can be mediated by macrophages and specific immunity. Yogurt feeding enhances murine defences against serovar Typhimurium (De Simone *et al.*, 1988, De Simone *et al.*, 1986) through several proposed mechanisms such as a) by increasing antibacterial activity of mononuclear cells against serovar Typhimurium, b) by specific IgA antibody production, c) by increasing the number of polymorphonuclear cells, as first line of defence of the host, against *Salmonella* infection, d) by increasing the proliferative response of both T and B cells. One or more of these mechanisms would produce a strong reduction of serovar Typhimurium growth in liver and spleen resulting in improved survival of animals treated with LAB.

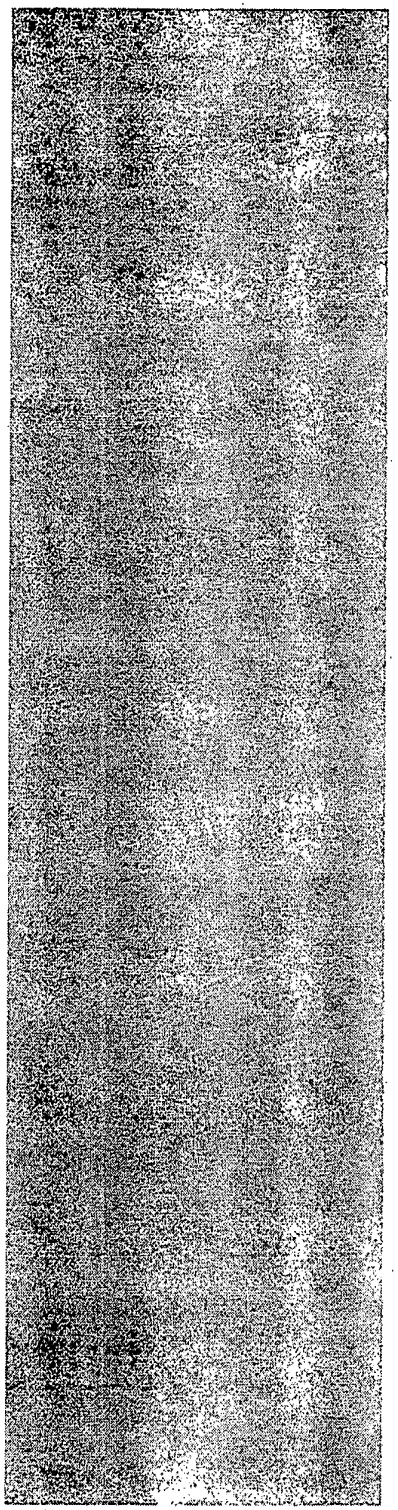
LAB contribute to the maintenance of colonisation resistance, mainly against *Listeria monocytogenes, Escherichia coli, Salmonella enteritidis* serovars Typhimurium and Enteritidis (Fernandes *et al.*, 1988, Chateau *et al.*, 1993). There is evidence from experimental and clinical studies which indicate that LAB administration could lead to significant changes in the intestinal microflora (Johansson *et al.*, 1993, Lidbeck and Nord, 1993). Futhermore it is well known that disturbances in the normal intestinal microflora lead to gastrointestinal disorders often resulting in diarrhoea (Johansson *et al.*, 1993). The ability of LAB to affect the systemic and mucosal immune responses suggests that these microorganisms would contribute to the recovery from infections. *Lactobacillus* GG has been shown to promote the recovery of children with rotavirus diarrhoea *via* augmentation of local and systemic immune response (Kaila, M. *et al.*, 1992). The exact mechanisms by which some LAB prevent enteric infections is unknown.

In virus infections of the gastrointestinal tract, there is a strong possibility that probiotics exert immunomodulatory mechanisms inducing a high level of specific secretory IgA which is a major immunological barrier against viruses. Yasui *et al.* (1995) showed protection against rotavirus, using *Bifidobacterium brevis*. Similar mechanisms may be responsible for protection against bacterial infections. Another possibility is that cytokine production may mediate immunostimulation (Solis and Lemmonier, 1993, Kitazawa *et al.*, 1994).

In human studies Link-Amster *et al.* (1994) demonstrated serum specific IgA against an attenuated *S. enteritidis* serovar Typhimurium given to volunters who consumed *L. acidophilus*.

Priming of GALT may be an important part of this protective mechanism. However, the competition for binding sites on epithelial cells resulting in competitive exclusión could be another way in which LAB increase the host's resistance to infection.

The mechanism of the host's response to probiotics is unclear. Much of the work indicates that probiotics exert a "barrier effect" against colonic pathogens, by the induction of specific IgA antibodies.



There are other studies in childhood diarrhoea using lactobacilli especially *L. acidophilus* and *L. casei* (Isolau[illegible] *et al.*, 1991, Saxelin *et al.*, 1998) which have been used to reduce *rotavirus*, *Salmonella* and *Shigella* infections. Attempts have beer[illegible] nade to use the non-pathogenic forms of certain organisms to induce bacterial interference against the virulent forms. Although th[illegible] competitive exclusion between non-pathogen and pathogen for the colonisation site could be effective, consumers may be reluctan[illegible] o ingest this type of preparation.

Protection against some enteropathogens can be obtained by vaccination, but at present oral vaccines using [illegible] acteria are not available.

There is now a renewed interest in the use of LAB as food additives to prevent diarrhoea. LAB are considere[illegible] by the Food Drug Administration (FDA) in USA as GRAS (Generally Regarded As Safe) microorganisms. These bacteria are o[illegible] n used in the prevention of diarrhoea of farm animals specially newborn piglets (Underdahl *et al.*, 1982). It is important to fi[illegible] d a treatment that will increase resistance to disease of newborn animals, including the human baby.

Oral stimulation with particulate antigens such as bacteria can induce a mucosal immune response, and LAB [illegible] ould enhance the mucosal immunity in the host.

In an attempt to analyze the protective effect of some LAB against a *Salmonella* infection we performed *in vi*[illegible] experiments using mice as the experimental model. LAB were administered prior to, or together with, the pathogen. The protecti[illegible] e capacity was determined by culture of the liver and spleen to detect the pathogen, and measuring the levels of anti-enterop[illegible] thogen s-IgA in the intestinal fluid by an ELISA test. If the LAB were effective, the invasive capacity of the pathogen should be su[illegible] essed at the intestinal level. Naturally these speculations are not valid in the case of the immunosuppressed host in which the react[illegible] e capacity of the system is diminished.

We studied the protective capacity against *Salmonella enteritidis* serovar Typhimurium infection of the followi[illegible] g LAB: *L. casei* CRL 431, *L. acidophilus* CRL 924 *L. rhamnosus* CRL 74, *L. delbrueckii* ssp. *bulgaricus* CRL 423, *Lactococcus lac*[illegible] CRL 526, *S. thermophilus* CRL 412 We observed that only some doses of *L. casei*, *L. delbrueckii* ssp. *bulgaricus* and *S. th*[illegible] *rmophilus* were able to protect against the pathogens (Table 1).

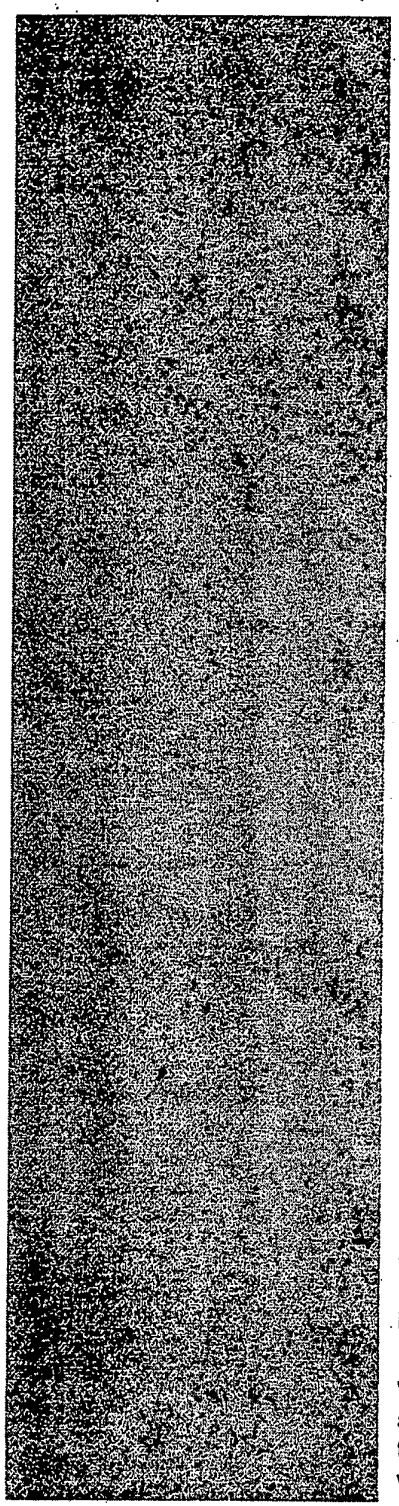


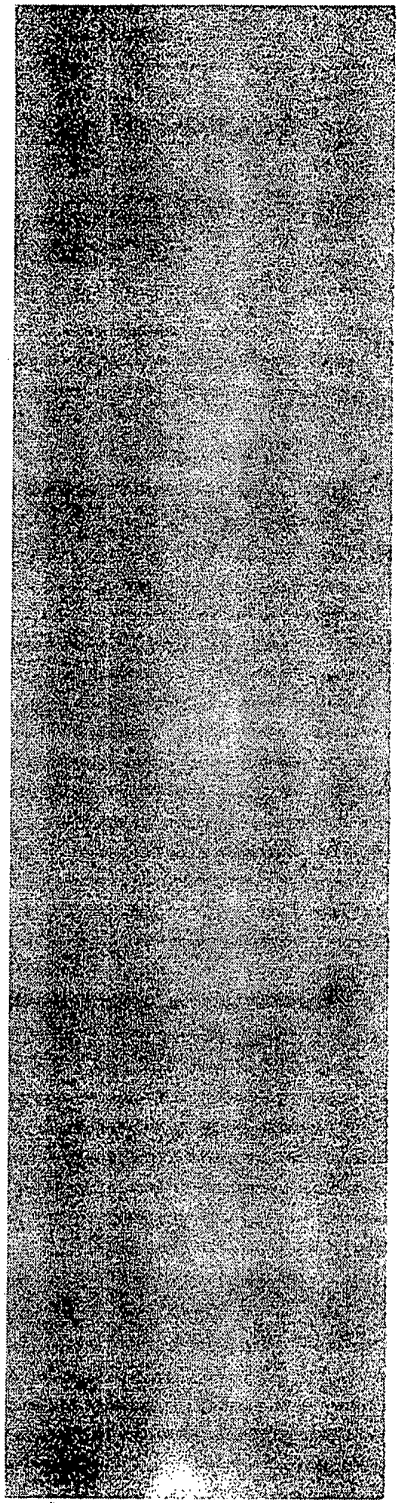
Table 1. Effect of the different LAB orally administered on the protection against *Salmonella enteritidis* subsp. *typhimurium* infection

Strains	Days of feeding	Log. N° of bacteria/organ
	2	2.2±0.5
L. rhamnosus	5	3.4±0.7
	7	3.4±0.4
Lac. lactis	2	3.2±0.1
	5	4.7±0.5
	7	3.3±0.2
L.acidophilus	2	3.1±0.8
	5	4.1±0.3
	7	4.9±0.4
L. casei	2	0
	5	4.2±0.5
	7	0
L. delb ssp. *bulgaricus*	2	1.3±0.2
	5	3.1±0.5
	7	0
S. thermophilus	2	1.1±0.7
	5	0
	7	0
L.plantarum	2	2±0.6
	5	1.5±0.5
	7	4.2±0.7
Control		5.4±0.4

Animals were treated with the LAB for 2, 5 or 7 consecutive days, at the end of each administration period they were challenged with 20 LD50 *Salmonella*. On the seventh day post-challenge, animals were killed and the liver and spleen were removed for colonization assays. Results represent the average of *Salmonella* number found in liver and spleen on the 7th day post challenge. Control are animals untreated with the LAB but challenged with *Salmonella*. Values are mean of n=6±SD.

We determined that only *L. casei* induced high levels of specific secretory IgA but the effect was dose dependent with lower doses being more effective than the higher one (Perdigón and Alvarez, 1992, Perdigón *et al.*, 1991, Perdigón *et al.*, 1990 a, 1990b). We also observed that the other LAB assayed induced an increase in the number of cells associated with the inflammatory immune response. We studied the increase of this inflammatory immune response by measuring the increase in intestinal permeability, for the antigen ovoalbumin orally administered at tolerogenic dose. We measured the levels of anti-ovoalbumin antibodies present in the serum by the ELISA test. We demonstrated that with LAB at doses that were not able to protect against *Salmonella*, intestinal permeability was increased. We are currently studying the mechanisms by which *L. delbrueckii* ssp. *bulgaricus* and *S. thermophilus*, which do not induce a specific s-IgA response, are able to protect against the pathogen.

We selected *L. casei* CRL 431 as the most appropriate strain with which to prevent enteric infection, and we examined the effect of administering it simultaneously with the pathogen, and after challenge, to study the therapeutic effect. In the latter case we determined the effect of repeated stimulation with *L. casei* (Perdigón *et al.*, 1993 a, 1993c). We found that *L. casei* administered simultaneously with the pathogen was ineffective indicating that *L. casei* was not able to inhibit the pathogen by competitive exclusion. We



demonstrated that *L. casei* administered during infection with *Salmonella* is beneficial for the host, but the ef ct was dose dependent. The increase in the synthesis of s-IgA (Alvarez *et al.*, 1998) was significant compared with the control when e time of administration was for periods of no more than 5 or 7 consecutive days. Thus the importance of the oral administration of a ppropriate LAB used as probiotic is mainly in the prevention of enteric infection; their use for therapeutic purposes is still limited.

Stimulation of the Secretory Immune System by LAB

Effect of LAB on the Gut Mucosa

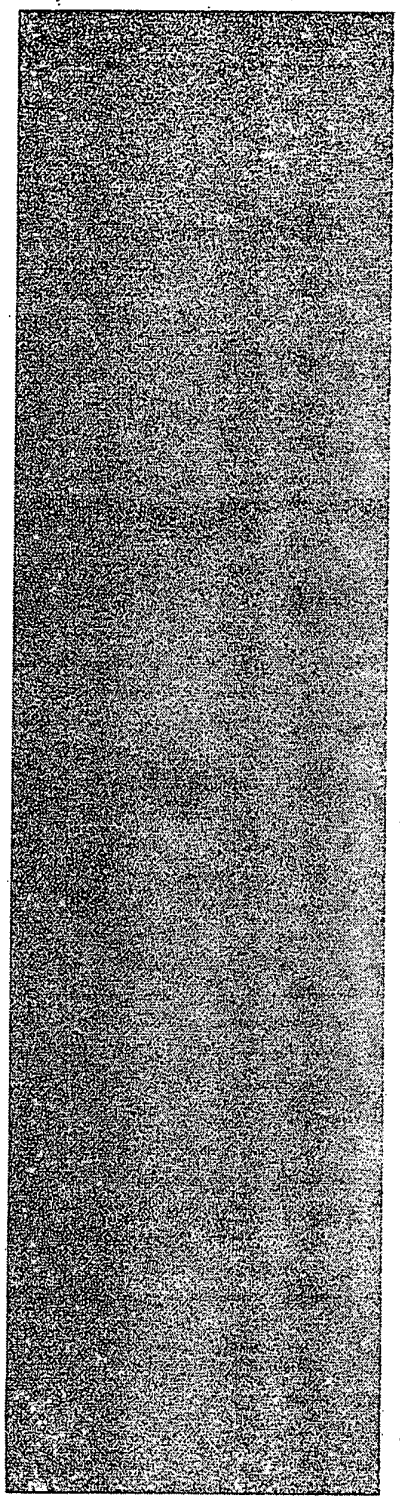
LAB are part of the normal microflora and inhabit the intestinal tract of humans, pigs, fowl and rodents. Many actors have been shown to affect the prevalence and distribution of LAB in the gastrointestinal tract. In humans it has been shown tha he stomach acidity reduces bacterial colonization, with the microflora mainly restricted to the distal small intestine increasing alo) its length into the colon where the largest microbial community develops.

The involvement of mucosal lymphoid tissue in host defense mechanisms has been extensively studied. GAI is the pivotal site for the induction of mucosal immune response in the gut, including the generation of oral tolerance.

The IgA antibodies can bind antigen and minimise its entry with a consequent reduction in inflammatory reac ns, which avoids a potentially harmful effect on the tissue. The induction of a local gut immune response may affect the secretor and absorptive functions of enterocytes as well as the motility of the gut, because the cytokines produced by the immune ce modulate several functions of the enterocytes both directly and indirectly *via* the recruitment of inflammatory cells. These may i duce the release of proinflammatory cytokines such as IL-1 (interleukin-1) and TNFa (tumour necrosis factor a), that increase thc ecretion of electrolytes and water into the human intestine (Perdue and McKay, 1993). Furthermore TNFa modulates the expression f the secretory component of IgA antibodies thereby regulating the transport of IgA across the epithelium (Kvale *et al.*, 1988 Other proinflammatory cytokines such as IFNg also induce the expression of Major Histocompatibility Complex (MHC) class II antig s on the enterocyte surface which contribute to the amplification of the local immune response. On the other hand IFNg (interfero g) and TNFa can directly mediate the killing of epithelial cells (Deem *et al.*, 1991). Thus the immune reaction can activate ente cytes in terms of cell mitosis and cytokine expression. Furthermore activated T lymphocytes from the lamina propria can enhance e proliferation rate of intestinal epithelial cells (Ferreira *et al.*, 1990). In addition cytokines released during the inflammatory immun response drive mesenchymal cells (fibroblasts, smooth muscle cells and endothelial cells) to produce eicosanids, other cyto nes and chemotactic and growth factors (Perdue and McKay, 1993). Another important amplifying mechanism of the intestinal infla mmatory response is the induction of gene expression for endothelial and macrophage adhesion molecules which act in synergy with c her chemoattractants to increase the infiltration of granulocytes, monocytes and lymphocytes into the inflammatory focus (Gundel and Letts, 1994).

How does the LAB modulate the gut immune response, especially the cytokine release which prevents an inc ease in the inflammatory immune response? Although it has been suggested that Gram-negative bacteria are the most e icient stimulus for driving the production of macrophage derived cytokines (Nicaise *et al.*, 1993), other reports have demonstrat l that Gram-positive bacteria, specifically LAB, can also induce proinflammatory cytokines. For example it has been described tha *L. acidophilus* induces the production of IFNa/b by murine peritoneal macrophages (Kitazawa *et al.*, 1992, Kitazawa *et al.*, 1994). *L delbrueckii* ssp. *bulgaricus* and *S thermophilus* have been shown to induce the production of IL-1b, TNFa and IFNg but not IF a and IL-2 (interleukin-2) by peripheral mononuclear cells (PBMC) from humans (Pereyra and Lemonnier, 1993) after ingestion of b teria in yogurt or sterile milk.

The age-related decline in the production of cytokines including IFNs is common and it has been demonstrat l that supplementing the diet of ageing mice with live *L. delbrueckii* ssp. *bulgaricus* and *S. thermophilus* restores completely the levels f IFNg and IFNa compared with the control animals (Muscettola *et al.*, 1994). There is also evidence that LAB, particulary *S. t rmophilus*, can induce production of proinflammatory cytokines such as IL-1 b, IL-6 (interleukin-6) and TNFa (Aattouri and Lemonni , 1995), and an oral preparation from *L. delbrueckii* ssp. *bulgaricus* stimulated the production of proinflammatory cytokines such IL-1 and TNFa (Popova



et al., 1993). De Simone *et al.* (1993) showed an increase in the NK cell cytotoxic activity by oral administration of *L. delbrueckii* ssp. *bulgaricus* and *S thermophilus*. Similar results were found using *L. acidophilus*, *L. casei* and *L. plantarum* (De Simone *et al.*, 1986). Even if the LAB administration under *in vivo* conditions strongly enhances the production of IFNg, this could be beneficial to the host, because that IFNg production is involved in mediating the increased resistance towards pathogens (De Simone *et al.*, 1988), as well as in the enhancement of the expression of the secretory component, contributing to an increase in the external transport of dimeric IgA. On the basis of previous results, LAB could modulate several functions of GALT. Available data also indicate that LAB such as *L. acidophilus* which can persist in the gastrointestinal tract, may act as adjuvants to the immune response (Link-Amster *et al.*, 1994, Schiffrin *et al.*, 1995).

The capacity of LAB to induce production of cytokines, means that they probably also affect other immune functions of the cells such as macrophages and granulocytes associated with mucosal sites. The possibility that LAB modulate the expression of cell-surface molecules that are involved in bacterial uptake by leucocytes should be investigated.

There are studies that show the importance of LAB administration in the preservation of intestinal integrity and stabilization of the gut mucosal barrier (Salminen *et al.*, 1988, Salminen *et al.*, 1996a). The intact intestinal epithelium with the normal intestinal microflora represents a barrier to antigens and other noxious substances from the gut lumen. In health this barrier is stable protecting the host and providing normal intestinal function. When either the normal microflora or the epithelial cells are disturbed by triggers such as dietary antigens, pathogens, chemicals or radiation, defects in the barrier mechanisms are induced with alteration in permeability, and mucosal inflammation (Isolauri, 1995).

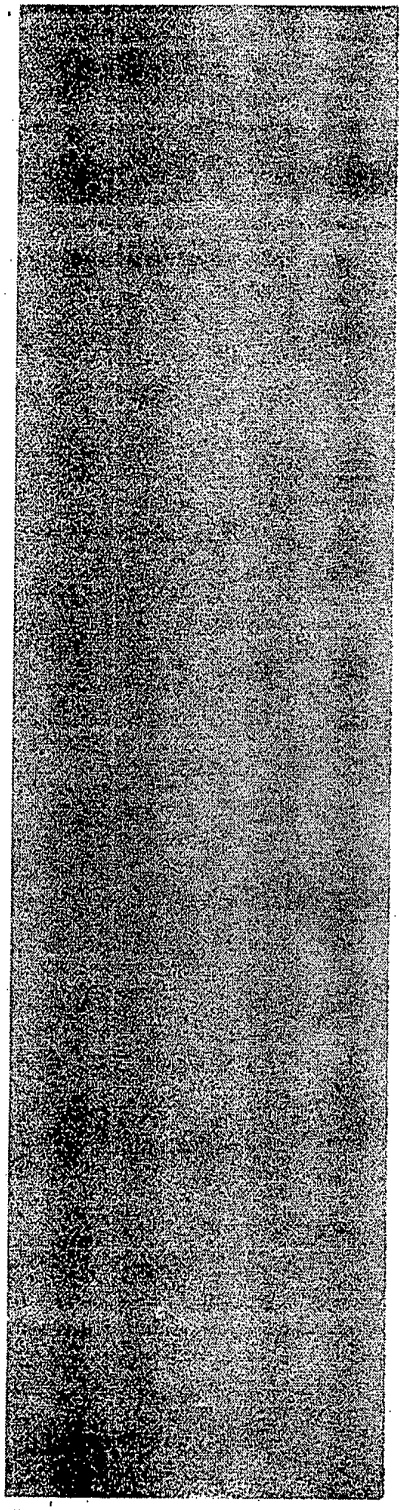
Even under physiological conditions, a quantitatively unimportant but immunologically important fraction of antigens bypasses the defense barrier. They are absorbed across the epithelial layer by tran-scytosis along two functional pathways. The main one is the degradative pathway which reduces immunogenicity, and diminishes the antigen load by more than 90%. A minor pathway allows the transport of antigens inducing an immune response. The integrity of the defense barrier is necessary to prevent inappropriate and uncontrolled antigen transport.

As a result of local intestinal inflammation, a greater amount of antigens may traverse the mucosal barrier and the routes of transport will be altered; this immunogenic stimulus favours allergic reactions (Fargeas *et al.*, 1995). Foreign antigens such as viruses, dietary antigens or pathogenic or non-pathogenic bacteria (LAB) can induce local inflammation in the intestinal mucosa. As a consequence of this inflammatory response the intestinal functions are modified and an increase in IgG^+ immune cells are observed, and translocation of the normal microflora may occur. Thus for successful use of LAB to improve secretory immune system functions, all of these previous considerations must be taken into account especially if, as is currently believed, the viability of the LAB strain is critical in determining the capacity of LAB to induce immune stimulation. LAB as probiotics have a great potential in the prevention and treatment of some clinical disturbances (Salminen and Deighton, 1992), however, each strain must be carefully selected and controlled studies in animals and humans must be performed to detected possible intestinal side effects.

In our laboratory, we use a mouse model and LAB which are frequently used in the food industry. We studied the effect of the oral administration of these LAB on: 1) side effects such as translocation of normal microflora to the liver and spleen, 2) which immune cells associated with the gut are activated, 3) effects on inflammatory immune response (Vintiñi *et al.*, 2000), 4) conditions required for use as an effective mucosal adjuvant (Alvarez *et al.*, 1998), 5) effect of oral administration of LAB on distant mucosal sites such as the bronchus (Perdigón *et al.*, 1999a), 6) determination of anti-LAB antibodies, 7) develop hypotheses on the possible mechanisms by which LAB could interact at the intestinal level and induce a secretory immune response. We found that only high doses of LAB can induce translocation of normal microflora to the liver and spleen.(Perdigón *et al.*, 1999 b, Perdigón *et al.*, 2000).

Study of the Immune Cells Involved in the Nonspecific Mucosal Immune Response

These tests were performed on histological slices from the small intestine. The immune cells involved in the inflammatory immune response (e.g. macrophages, neutrophils and eosinophils) were studied for different LAB over different periods of administration (2, 5



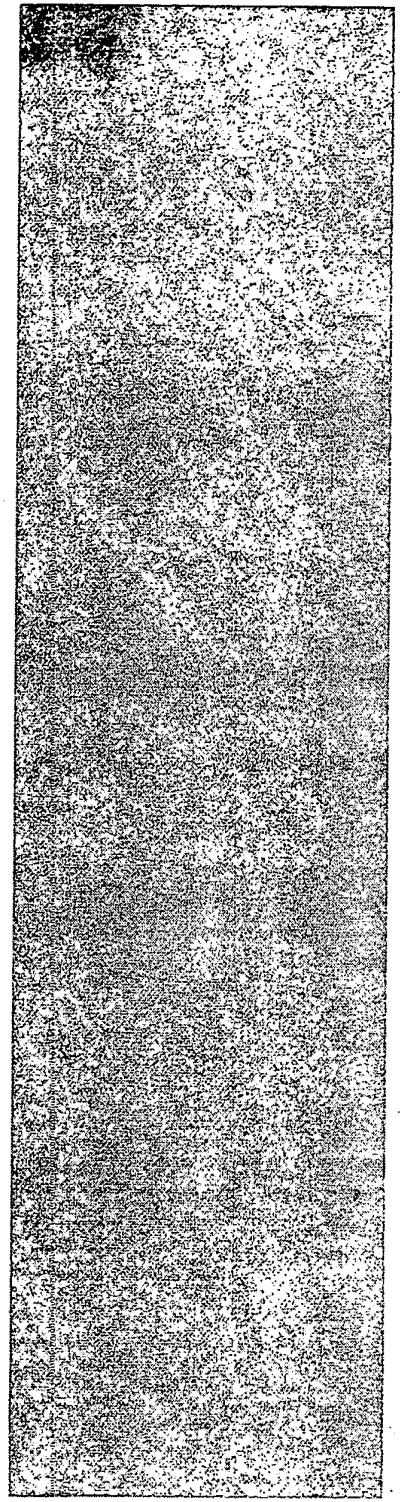
or 7 days). Only some of the LAB such as *L. rhamnosus* and *Lactococcus lactis* for 5 and 7 days increased t[illegible] number of macrophages, neutrophils and eosinophils and the inflammatory immune response. This effect was also obs[illegible]ved in histological slices from the small intestine stained with haematoxilin-eosin where oedema within the villi was observed. We als[illegible]etermined the inflammatory immune response by measuring of IgG secreting cells and $CD8^+$ T cells present on the lamina [illegible]opria of the small intestine as a marker of inflammation.

The number of IgG^+ B cells and $CD8^+$ T cells was assessed by direct immunofluorescence using monospeci[illegible] and monoclonal antibodies respectively. With the exception of *L. plantarum* fed for 2 days, none of the other LAB assayed inc[illegible]ased the number of IgG^+ B cells or $CD8^+$ T cells. These results are shown in Table 2.

Table 2. Effect of oral administration of LAB on IgG^+ B cells and $CD8^+$ T cells associated with the lamina propria o[illegible]he small intestine

Strains	Days of feeding	IgG^+ B cells (Number/10 fields)	$CD8^+$ T cells (Num[illegible]r/10 fields)
L. rhamnosus	2	11±1	18±4
	5	22±1	12±4
	7	10±1	17±5
L. acidophilus	2	48±3	64±6
	5	33±2	40±6
	7	25±1	39±5
L. casei	2	31±1	50±5
	5	39±3	55±6
	7	32±1	47±5
L. delb. ssp. bulgar.	2	30±3	35±7
	5	23±3	41±6
	7	26±1	50±7
S. thermophilus	2	33±3	49±5
	5	20±3	31±5
	7	19±2	38±7
Lac. lactis	2	52±5	41±6
	5	36±3	54±5
	7	18±1	38±5
L. plantarum	2	71±1	107±8
	5	23±2	38±7
	7	12±2	33±6
Normal control		45±1	58±5

Histological slices from the small intestine were prepared at the end of each period of administration. The IgG^+ B [illegible]lls and $CD8^+$ T cells were determined by direct immuno-fluorescence using monospecific[illegible]y chain and monoclonal anti-CD8 antibo[illegible]es labeled with fluorescein, respectively. Values are mean of $n = 5 \pm SD$. ** $P < 0.01$ * $P < 0.05$. Controls are mice without LAB a[illegible]inistration.



Effect of LAB on the Specific Mucosal Immune Response

In this study we analyzed the number of IgA^+, IgM^+ B cells and $CD4^+$ T cells associated with the lamina propria of the small intestine. The immune cells were determined on histological slices by direct immunofluorescence. The number of IgM^+ cells was studied to determine if LAB interaction induced modification in the number of IgM^+ cells by induction of a switch from IgM^+ to IgA^+ as a result of the cytokines released by $CD4^+$ activated T cells.

The number of IgM^+ cells was increased by 2 days of feeding *Lactococcus lactis*, 2 and 5 days of *L. acidophilus,* and 2 and 7 days of *L. plantarum*. This could mean that no switch was induced and that LAB stimulation only evoked the clonal expansion of those cells present on the lamina propria. We observed that all the LAB assayed were able to increase the number of IgA^+ cells favouring a good intestinal mucosal immune response. The effect was dose dependent. With the exception of *L. casei* the increase of IgA^+ cells was not correlated with an increase in CD4+ T cells.

L. casei was the best inducer of a specific gut immune response accompanied by an increase in IgA^+ and $CD4^+$ T cells. Therefore, we attempted to answer the following questions: a) how long does the immune response last and, b) when a booster is given to maintain an optimal mucosal state is it able to prevent enteric infection?

We demonstrated that: a) the importance of the size of the dose of *L. casei* that was administered for 2 days to obtain an effective protection against *Salmonella* infection, b) that the number of IgA^+ cells must be slightly increased to avoid harmful effects, as was shown in coeliac disease (Brandtzaeg *et al.,* 1993), c) to have a good mucosal immune response the rate between $CD4^+/CD8^+$ should be maintained at 50 to 50 similar to the control values, d) boosting with a single dose (10^9 cells) each 15^{th} day is necessary to protect against *Salmonella* infection (Perdigón *et al.,* 1995 a, Alvarez *et al.,* 1998).

Effect of LAB on IgA^+ Cells Associated With the Bronchus

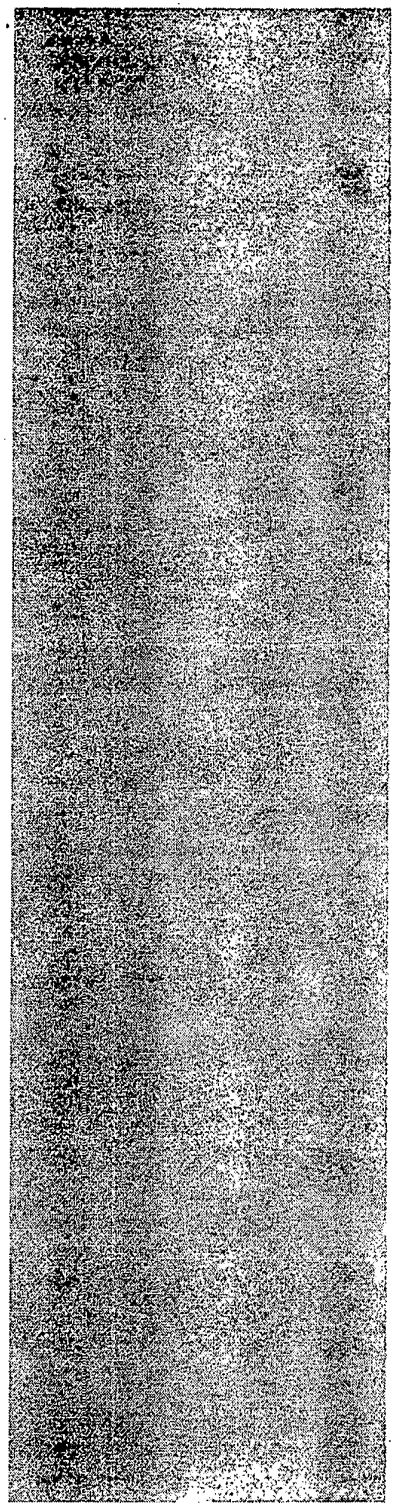
We determined whether the oral administration of LAB induced an increase in the IgA^+ B cells, in distant sites such as the bronchus. We determined the number of IgA^+ cells present in the bronchus for each LAB and after 2, 5 or 7 days of feeding was determined. All of the LAB with the exception of *L. acidophilus,* increased the number of IgA^+ cells in the bronchus (Perdigón *et al.,* 1999a). The effect was dose dependent. The oral administration of LAB increased the number of IgA^+ cells entering into the IgA cycle repopulating other mucosal sites such as the bronchus. This observation is important, because the most frequent portal of entry for pathogens is the respiratory tract; oral ingestion of LAB could protect the respiratory mucosa.

Study of the Anti-LAB Immune Response

This study was performed to determine if the LAB that interact at different levels of the intestinal tract (Peyer's patches or epithelial cells) were processed and presented as antigen, inducing specific anti-LAB antibodies. These antibodies were determined in the intestinal fluids by an ELISA test. We observed that *L. casei, L. rhamnosus, S. thermophilus* and *L. plantarum* induced specific IgAantibodies against their epitopes, meaning that those microorganisms were processed and presented as antigen by the immune cells associated with the gut mucosa. However, we do not know the exact role played by these anti-LAB antibodies, neither why not all the LAB assayed were not processed as antigen. We think that the pathway of internalization of LAB to interact with the immune cells associated with the intestine is important in understanding these results.

The Possible Mechanisms of Interaction of the LAB With the Gut.

Taking into account the results obtained on IgA^+ cells present in the small intestine and in the bronchus, and observations concerning



CD4+ T cells and anti-LAB antibodies, we suggested the following mechanisms of LAB interaction with the s ll intestine (Perdigón *et al.*, 1999b).

a) *L. casei* and *L. plantarum* would interact at the Peyer's patches level because these microorganisms indu cell migration and increase the IgA+ cell cycle. As CD4+ T cells are enhanced in the lamina propria of the intestine, this would r an that CD4+ T cells enter into the IgA cycle and repopulate the lamina propria. The interaction in Peyer's patches is the only way induce the migration of T cells. These LAB were processed as antigen because anti-LAB antibodies were detected.

b) *L. rhamnosus, S. thermophilus, L. delbrueckii* ssp. *bulgaricus* and *Lactococcus lactis* would interact with t epithelial cells of the small intestine or with the epithelial cells associated with follicle FAE in Peyer's patches. This possibility is su ported by the fact that these LAB increased the number of IgA+ cells at intestinal and bronchus level but not CD4+ T cells. The IgA cle can also be increased by stimulation of the IgA+ cells from the mesenteric node (Weiner, 1997). The interaction with epit ial cells can induce activation of T cells by cytokines released from them, inducing production of other cytokines from T cells ass iated with the lamina propria. At the epithelial cell level, the LAB may or may not be processed as antigen.

c) *L. acidophilus* would induce only a local gut immune response because the only parameter increased was testinal IgA+ cells. Because of the ecological niche which it occupies, *L. acidophilus* would also be expected to interact with the ithelial cells of the large intestine.

We demonstrated that the LAB can interact at different levels in the small intestine (Peyer's patches, FAE or th the epithelial cells), or with the large intestine (Perdigón *et al.*, 2000). The different ways of interaction might explain why the muc al immunostimulation by LAB is not the same for all of them. In some cases they induce an inflammatory immune response and in hers a specific mucosal immune response. The immune stimulation by LAB cannot be generalized for genera or species; this proper may be strain specific.

Even if our hypothesis of interaction of the LAB with the gut is confirmed, other important questions should b nswered:

a) Why, if all the LAB have the same muramyldipeptide antigen, mucosal stimulation is not the same with all them?

b) What are the conditions required by LAB to bind to M cells of Peyer's patches and to induce a complete in une response?

c) Is the production of specific anti-LAB IgA an advantage or not?

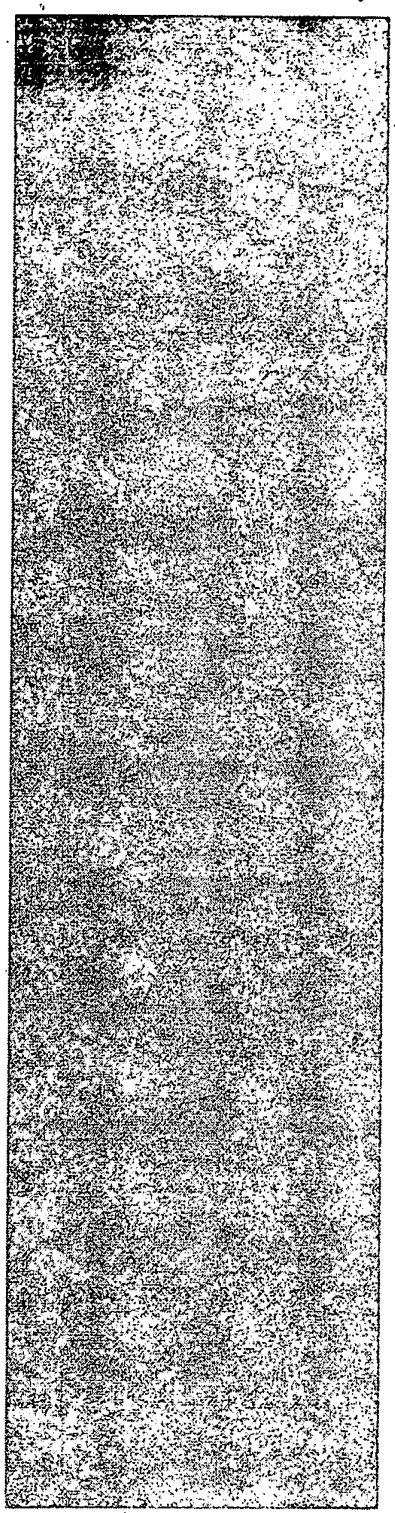
d) Is the site interaction of the LAB with the intestine restricted to the ecological niche?

e) Can the selection of a strain of LAB with immunopotentiation capacity, to be used as a probiotic, be compr nised by adverse side effects?

f) Can a strain of LAB with immunological activity, be used as a mucosal adjuvant or as a vehicle for oral vac es? This last aspect will be discussed in the following section.

LAB as Vaccine Vectors

The existence of a common mucosal immune system can be exploited to design vaccines capable of protecti g mucosal surfaces that are less accessible to mucosal immunization. Accoding to Jennings *et al.* (1998), an ideal mucosal vaccine s uld: a) promote an effective contact of the antigen with the immune system; b) stimulate specific humoral and cell-mediated imm ity responses; c) elicit



a long-lasting protection after a single-dose in early infancy; and d) be stable and non-toxic.

Recent work has highlighted the potential of lactic acid bacteria (LAB) as antigen presenting vehicles suitable for mucosal administration (Mercenier, 1999; Wells *et al.*, 1995, 1996). They have no recorded toxic or pathogenic activity (i.e.: no harmful effect was observed after subcutaneous or oral administration of mice with up to 10^9 live cells of *Lactococcus lactis*. The studies for the safety assessment of LAB have been summarised by Salminen *et al.* (1996b). On the other hand, some species of lactobacilli are maintained transiently or are able to colonise the gut mucosa, and certain strains have intrinsic adjunvant activity which may promote the immunogenicity of heterologous antigens (Perdigón *et al.*, 1995 b; Pouwels, 1996). The dairy industry has a long experience with these cultures, and their preparation on a large scale for a live LAB vaccine would be cost-effective (feasible and a cheap alternative to other delivery systems).

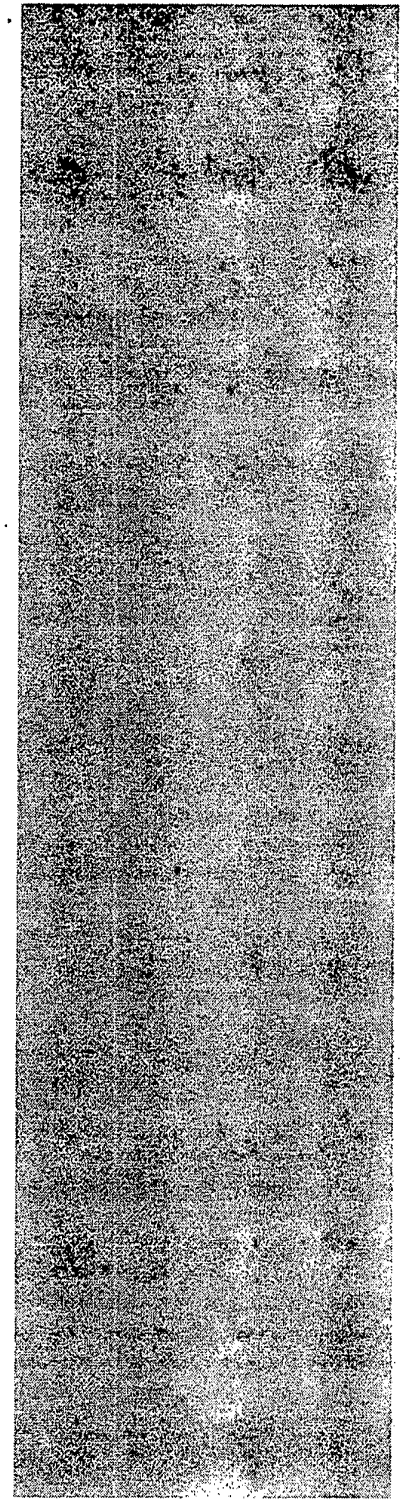
Several genera of LAB are being tested as vaccine delivery vehicles, but here attention will only be given to the work with *Lactococcus lactis* and certain species of lactobacilli. To develop LAB as live vaccines, they have to be genetically transformed. Protocols for the transformation of LAB have been optimised and last-generation food-grade vectors for expression, secretion, and surface- anchoring of antigens are available (Klaenhammer, 1995, Kleerebezem *et al.*, 1997, Kok, 1996). Limited progress has been made within the genus *Lactobacillus* since some species are refractory to transformation and often show a strong strain-specificity in gene expression. Production of recombinant proteins (antigen) in this genus can be achieved in three different ways: intracellulary, extracellulary and surface-bonded. Selecting one of these alternatives is of paramount importance because the size, nature, molecular weight and organisation of the antigen may affect humoral and cellular immune responses. Several studies showed that recombinant *Lactococcus lactis* strains are suitable for oral administration to stimulate responses at mucosal surfaces: a protective humoral response was elicited against a bacterial antigen (tetanus toxin) after nasal or oral immunisation of mice (Wells *et al.*, 1993).

Highly efficient expression vectors have been developed which successfully expressed and secreted heterologous fusion proteins in *Lactococcus*. In these vectors, expression of heterologous proteins is driven by either a strong constitutive promoter (i.e.:lactococcal phage P1 promoter) or an inducible system (*Escherichia coli* bacteriophage T7 RNA polymerase or nisin expression system), while secretion is driven by signal sequences known to be functional in several LAB (i.e.: signal sequences from PrtP, a cell-envelope associated proteinase found in *Lactococcus lactis* SK11 and *L. paracasei* subsp. *paracasei*, and from usp45, a secreted protein found in *Lactococcus lactis*). Tetanus toxin fragment C (TTFC), the B subunit of cholera toxin (CT), or protective epitopes (i.e.: the gp41E) can be expressed and presented to the immune system in an immunogenic form (Agren *et al.*, 1999) In *Lactococcus lactis*, up to 22% of soluble TTFC was expressed intracellularly and about 2.9 mg of TTFC was secreted (Wells *et al.*, 1993).

Live lactococci have been developed as mucosal vaccine delivery vectors for recombinant proteins associated with microbial virulence. It has been shown that *Lactococcus* vaccines elicit protective antibody and cell mediated immune responses in the host after either parenteral or mucosal immunization. Intranasal (i.n.) or oral administration of recombinant *Lactococcus lactis* expressing TTFC to C57 BL/6 mice elicited mucosal s-IgA and serum IgG responses (primarily of the IgG1 and IgG2a subclasses), which suggested involvement of both Th1 and Th2 $CD4^+$ T cell activity. In addition, secretory antibody responses in the lung and nasal tissues were elicited after intra-nasal inoculation in the presence of the adjuvant (Norton *et al.*, 1997). Further, the vaccine elicited protective immunity against lethal challenge of mice with tetanus toxin. Both killed and live recombinant strains induced similar immune responses and no requirement for either colonization or invasion of the mucosa was observed (Robinson *et al.*, 1997; Wells, 1996).

Recombinant lactococci can also deliver cytokines to the immune system. Secretion of recombinant murine interleukin-2 (mIL2) or mIL6 (shown to be the most effective terminal differentiation factor for IgA-committed B cells to become IgA-producing cells, in both human and murine systems) were achieved in *Lactococcus lactis* using the secretion signal leader of the lactococcal usp45. The rIL-2 showed the same specific biological activity as mIL-2 (Steidler *et al.*, 1998a). An enhanced immune response against TTFC was observed in mice immunised with live recombinant *Lactococcus lactis* strains which expressed both interleukins and TTFC.

Progress in the use of *Lactobacillus* strains as live vaccines is limited (Pouwels *et al.*, 1996). Several strains have been evaluated for



their ability to produce and secrete the B subunit of cholera toxin (CTB), alpha-amylase, or an epitope from h man immunodeficiency virus (gp41 protein) under the control of a set of expression or expression/secretion signals from various lact acid bacteria (Hols *et al.*, 1997, Piard *et al*, 1997). The capacity to secrete heterologous protein varied between different species, t highest level being detected in *L. plantarum* NCIMB 8826 (levels as high as 10 mg l-1 of the M6-gp41 fusion protein were secre l; Hols *et al.*, 1997). Secretion of CTB molecules by this strain was also efficient, but no folding of the B toxin subunits in pentam , and therefore GM1 ganglioside binding activity, was found.

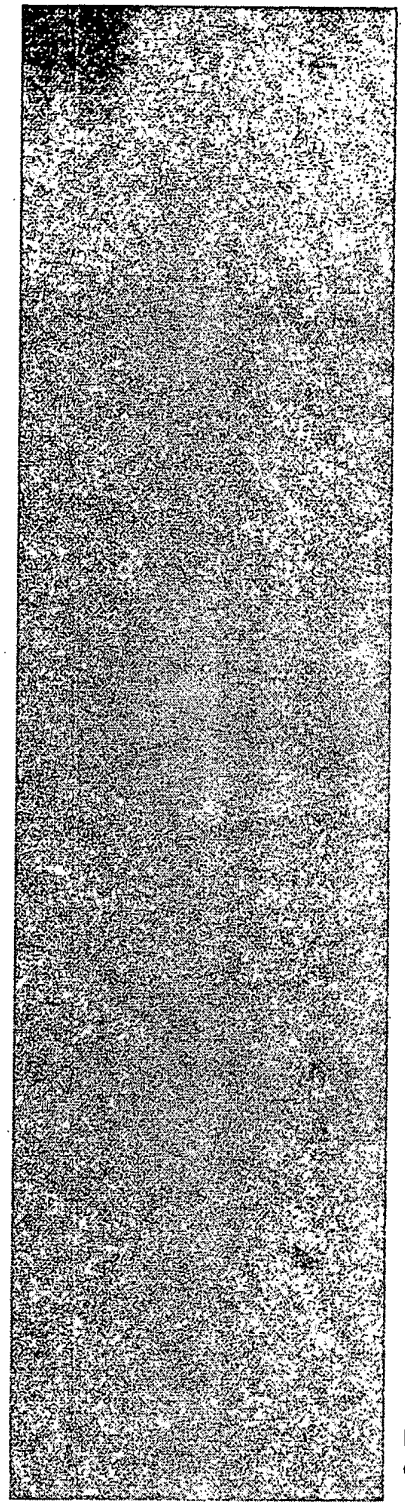
The development of new expression systems designed for cell surface display of chimeric antigens on LAB, ing signals of the lactococcal usp45 secretion peptide and of the cell wall anchoring of protein A from *Staphylococcus aureus* protein M6 from *Streptococcus pyogenes*, have been recently described. Streptavidin monomers fused to protein A (Steidler *al.*, 1998b) and several M6-fusion proteins (Hols *et al.*, 1997) have been successfully expressed, secreted and anchored to the cell ll of several LAB. The M6-fusion proteins were successfully secreted into the growth medium, at levels of 5 mg/l, by recombinant *La tococcus lactis* cells containing a low copy plasmid (about 10^5 molecules per cell), even though most of M6-fusion recursors acc ulated in the cytoplasm. The highest amount of a heterologous protein secreted by LAB has been reported by Savijoki *et al.* (1997); 8 mg/ml of a reporter gene (ß-lactamase) were secreted into the growth medium by S-layer signals from *L. brevis*, both in *Lactoco us lactis* and *Lactobacillus brevis*. Application of this efficient system in antigen presentation shows considerable potential r enhancement of immune responses.

Finally, protein A is also well known for its strong binding to IgG subclasses. Recently, it has been shown tha he enzymatically active toxin A1 subunit fused to two recombinant Ig-binding domains of staphylococcal protein A was primarily targ d to B cells with comparable adjuvant ability to that of native cholera toxin (Agren *et al.*, 1999). Furthermore, it was not toxic. e potential use of this system to target antigen delivery by LAB vaccines to B cells deserves special consideration.

Conclusions

In the selection of immunomodulating strains of LAB capacity, it is important to know if they induce good muc al immunostimulation without inducing side effects such as bacterial translocation or a strong inflammatory immune response which can alter intestinal permeability. It is also necessary to control the levels of IgA; these can be influenced by the dose administere . Depending on the LAB interaction with the intestine (Peyer's patches, FAE or epithelial cells) the immune response obtained wi be different at different mucosal sites. In spite of the multiple screening needed to check immunopotentiator activity of LAB, we only n predict their behaviour in the complex interactions within the intestinal ecosystem and their influence on the immune cells. hus if some LAB induce IgA^+ B cell and $CD4^+$ T cell migration we can predict that this LAB would have local and systemic effe s, but if the LAB do not induce an increase in the IgA migration their action would be only at the gut level and could be used to incre e intestinal mucosal immunity.

The development of effective mucosal vaccines relies almost entirely on our understanding of the mucosal im une system. However, much remains to be learnt about the cellular and molecular mechanisms involved in the control of the mucos immune system: i.e., antigen presentation, IgA B-cell differentiation, T-cell regulation, and development of long term immunologica memory. Although there are many reports characterising LAB as delivery systems for oral antigen administration, the development of combinant lactic acid bacteria vaccines is still in its early stages. Advances are expected in the construction of improved delivery s tems for oral administration of antigens that are immunogenic and targeted to specific areas or cells in the gut. The nature of the vehicle delivery system plays an important role in the type of cells induced with subsequent mucosal or systemic antibody res onses. Therefore, more appropriate basic knowledge has to be acquired about the type of antibody responses (e.g., Th1 or Th2), the ature and roles of cytokines, or phagocytic cellular functions elicited by recombinant lactic acid bacteria. This information can p ide the scientific foundation that will be useful in designing rational guidelines for development of efficacious LAB live vaccine Finally, before these LAB vaccines are used for humans, the pharmacokinetic properties and rigorous clinical trials are necessary assess their efficacy and safety.



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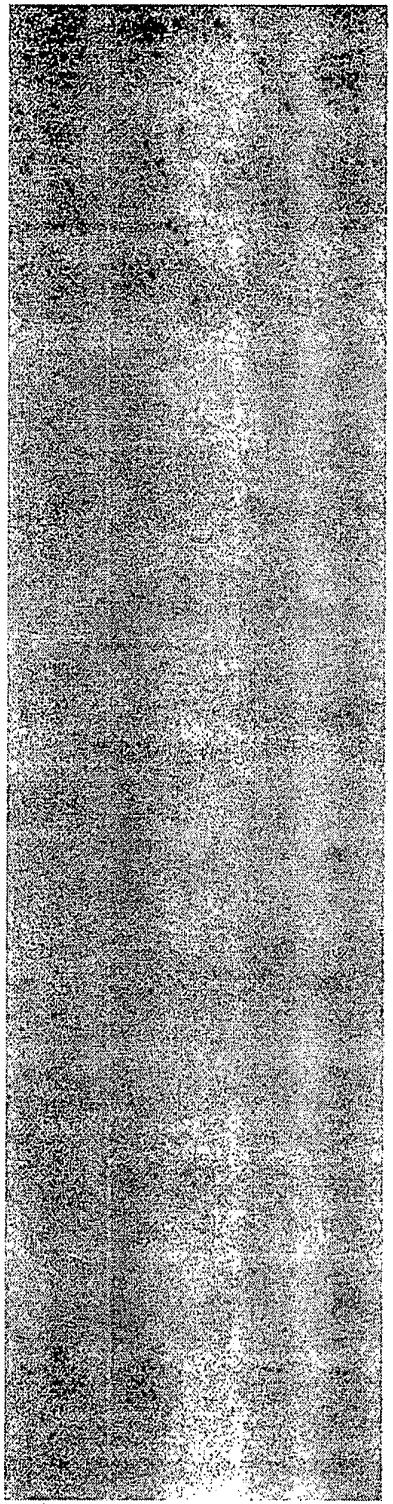

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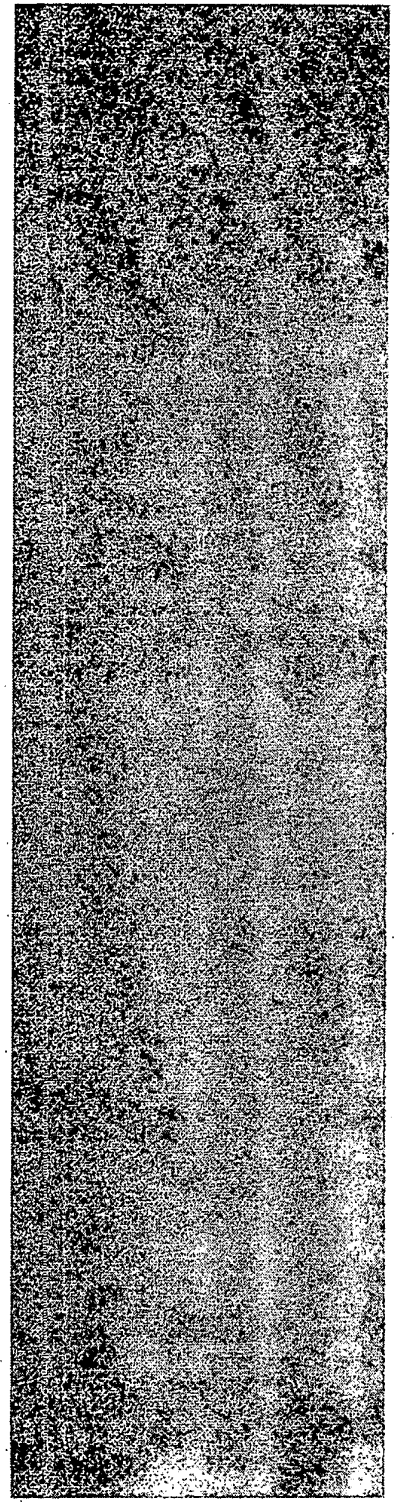

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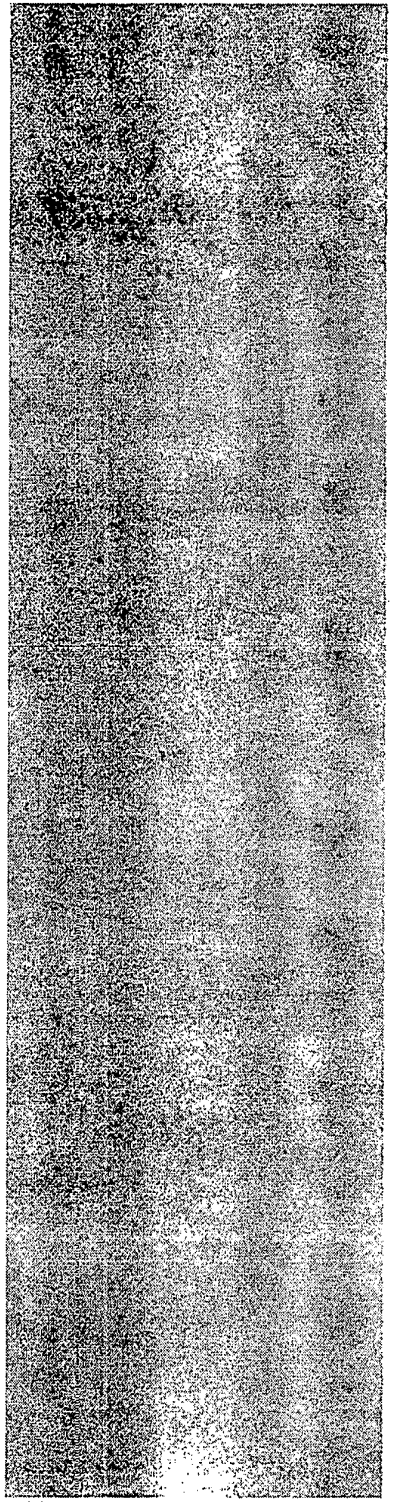

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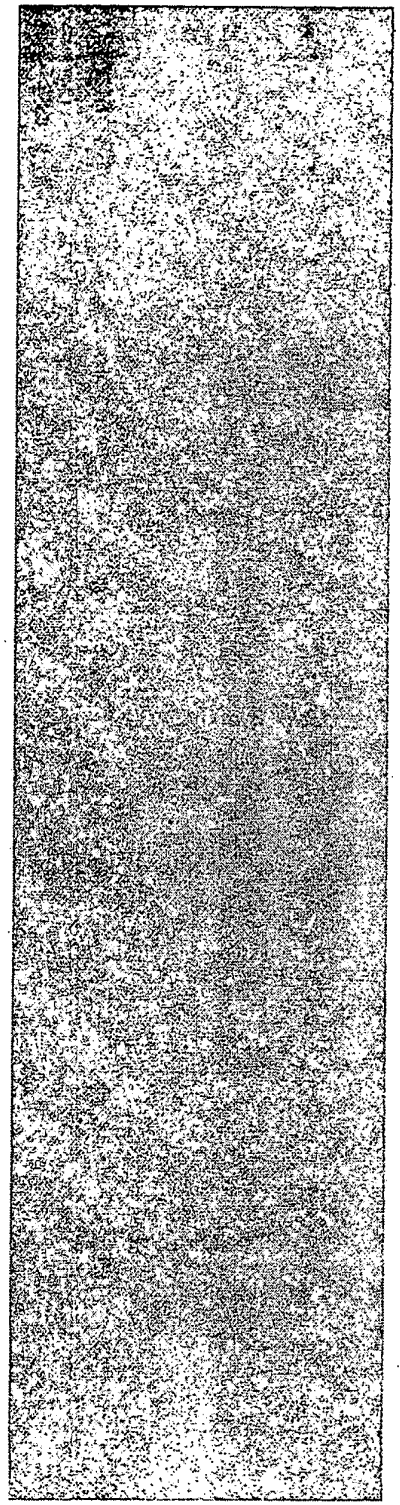

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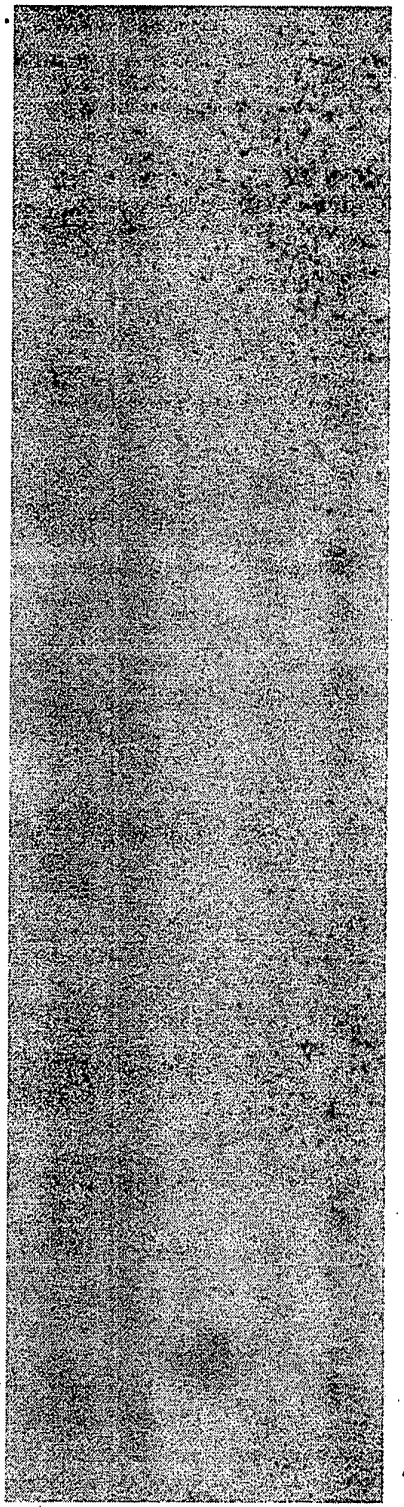

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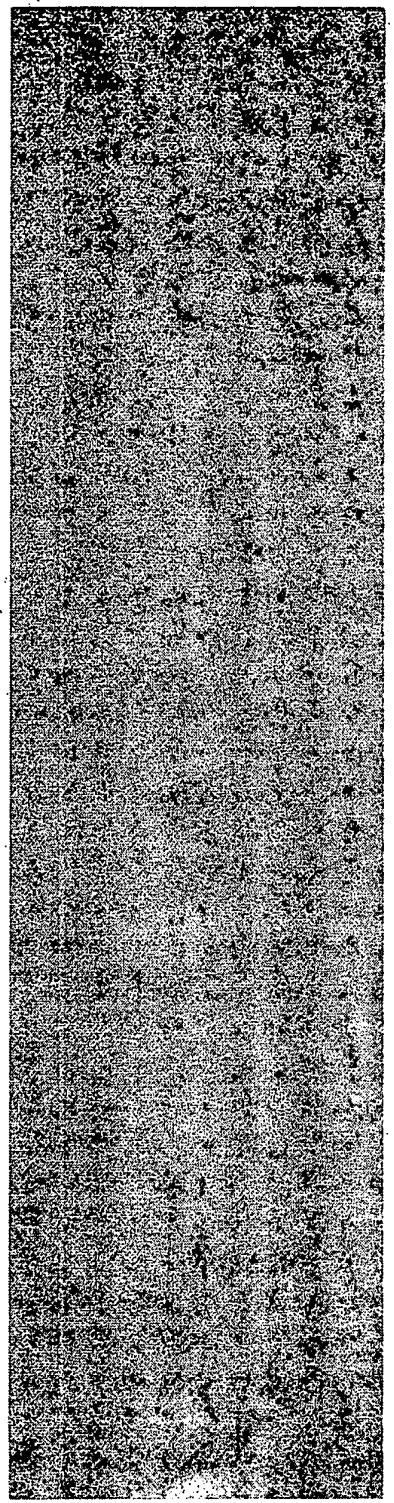

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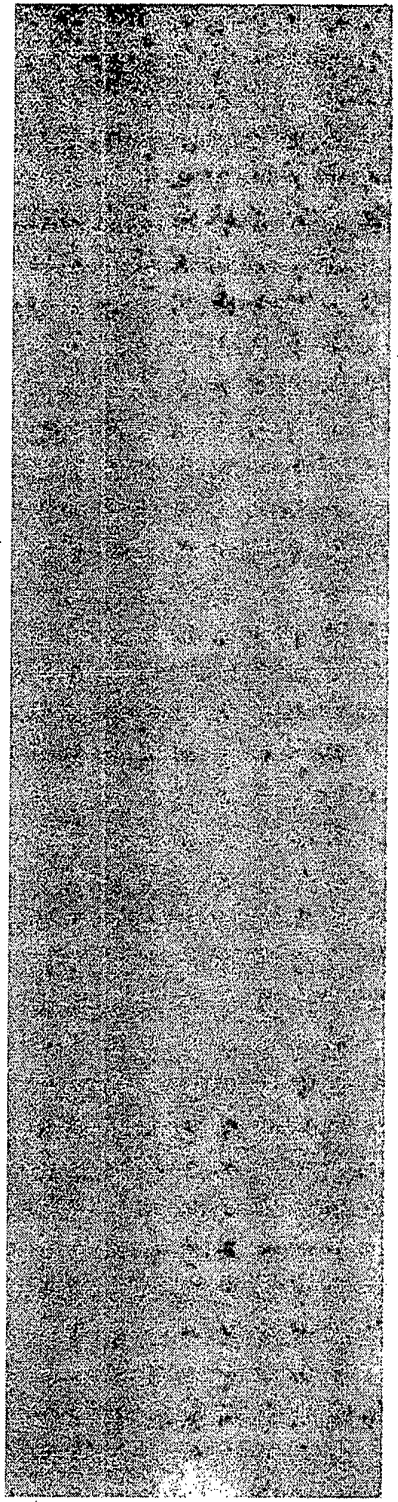

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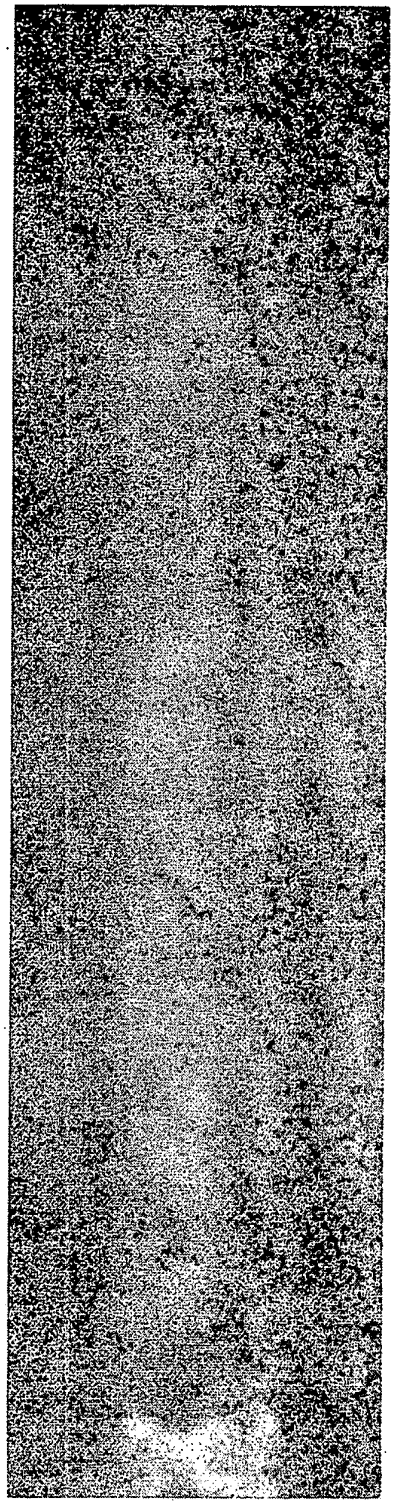

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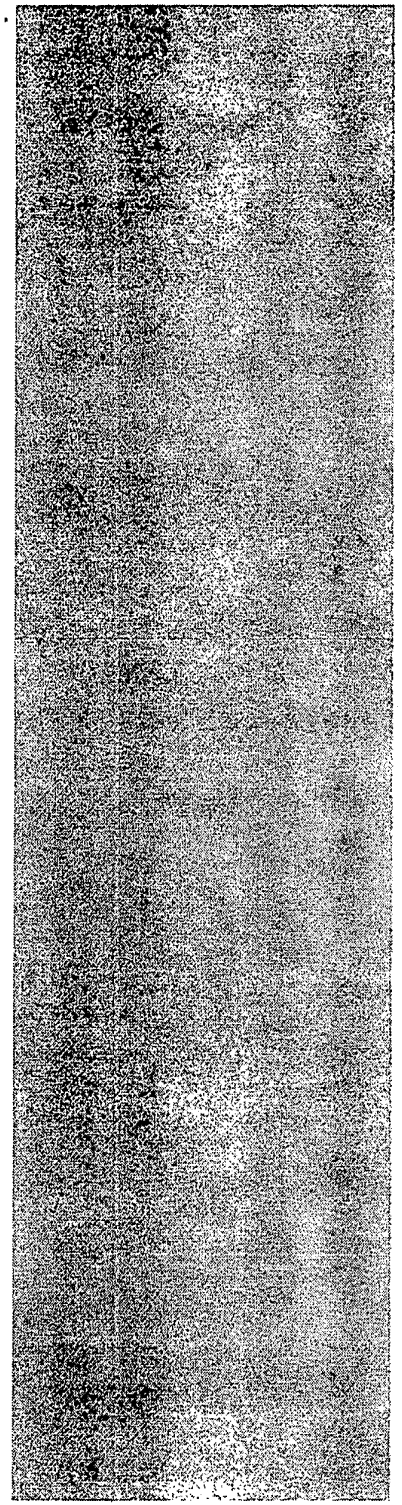

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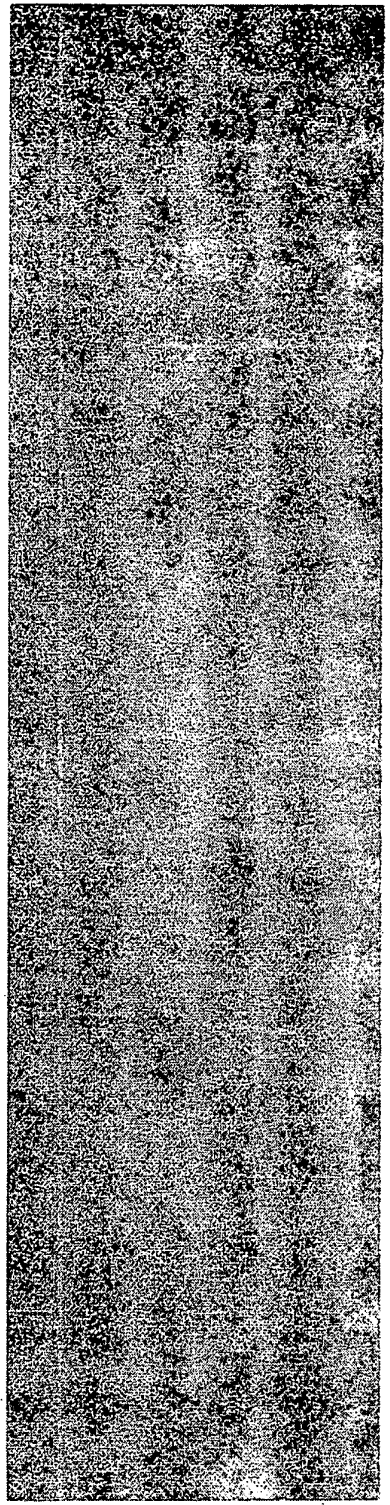

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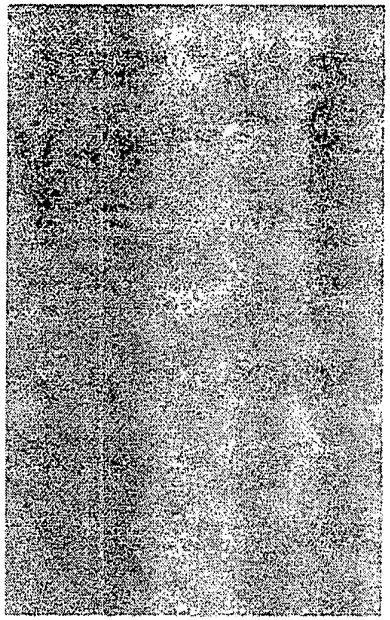

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Gut Mucosal Immunostimulation by Lactic Acid Bacteria

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Key words: mucosal immune cells, intestine, Lactic Acid Bacteria

ABSTRACT: The beneficial properties of lactic acid bacteria (LAB) on human health have been frecuently demonstrated. The interaction of LAB with the lymphoid cells associated to the gut to activate the mucosal immune system and the mechanisms by which they can exert an adjuvant effect is still unclear, as well as if this property is common for all the LAB. We studied the influence of the oral administration of different genous of LAB such as *Lactobacillus casei*, *L. acidophilus*, *L. rhamnosus*, *L. delbrueckii* subsp. *bulgaricus*, *L. plantarum*, *Lactococcus lactis* and *Streptococcus thermophilus*. We determined if the LAB assayed were able to stimulate the specific, the non-specific immune response (inflammatory response), or both. We demonstrated that all the bacteria assayed were able to increase the number of IgA producing cells associated to the lamina propria of small intestine. This effect was dose dependent. The increase in IgA^+ producing cells was not always correlated with an increase in the $CD4^+$ T cell number, indicating that some LAB assayed only induced clonal expansion of B cells triggered to produce IgA. Most of them, induced an increase in the number of cells involved in the inflammatory immune response. $CD8^+$ T cell were diminished or not affected, with exception of *L. plantarum* that induced an increase at low dose. This fact would mean that LAB are unable to induce cytoxicity mechanisms.

We demonstrated the importance in the selection of LAB to be used as gut mucosal adjuvant. The different behaviours observed among them on the gut mucosal immune response, specially those that induce inflammatory immune response, show that not all the LAB can be used as oral adjuvant and that the beneficial effect of them can not generalized to genous or specie. The immunoadjuvant capacity would be a property of the strain assayed.

Introduction

The intestinal microenvironment is extremely complex. It contains normal microflora and immune cells associated to mucosa surfaces. The indigenous microflora of the digestive tract consist of autochthonous microorganisms which largely stay in the host and the transient one. These microorganisms play a role in the development and maintainance of the activity of the immune system associated to the gut-associated-lymphoid tissue (GALT); that includes IgA, $CD4^+$, $CD8^+$ T cells and intraepithelial lymphocyte (IEL) activation. It has been demonstrated that obligate anaerobic Gram positive bacteria not only provide the stimulus for populating the lamina propria with IgA cells but also for the increase of cellularity in the intraepithelial compartment (Moreau *et al.*, 1978; Klasen *et al.*, 1993). It was suggested that the gut flora can be modified by the ingestion of certain non pathogenic microorganisms called

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"probiotics" (Fuller, 1992). A group of these bacteria are the lactic acid bacteria (LAB), which can influence the intestinal microenvironment and to produce beneficial effects in the host.

Lactic acid bacteria and the fermented products containing these microorganisms can decrease blood cholesterol level, induce antitumour immunity and secretory immune system stimulation (Agerback *et al.*, 1995; Matzusaki *et al.*, 1985; Schiffrin *et al.*, 1995; Sanders, 1993; Perdigón *et al.*, 1998).

The use of LAB fermented milks as oral adjuvant has been suggested in the prevention against enteric infection (Saavedra *et al.*, 1994; Isolauri *et al.*, 1991). However, its use in human being is still limited, because there is none or poor information about the mechanisms involved to exert beneficial effects on the host (Halmiton-Miller, 1996).

Although direct transposition of results from animals studies to human, is not always possible, they may serve to give an indication of the potential benefits available of these probiotic bacteria and also give information about how the probiotics work, and what immune cells are activated.

In previous studies we have demonstrated that different LAB are able to activate the systemic immune response (Perdigón *et al.*, 1986, 1988) and that *L. casei* was able to induce a secretory immune response depending of the dose administered (Perdigón *et al.*, 1991, 1995).

The aim of this work, was to study how the ingestion of LAB as mucosal adjuvant can influence on the activation of the immune cells associated to the gut, and if the oral immunoadjuvant property described for the LAB assayed can be extrapolated to the genous or specie.

Materials and Methods

Animals

Female BALB/c mice weighing 25-28 g of 6 weeks age were obtained the random-bred colony kept in our department at the Institute of Microbiolgy. Each experimental group consisted of 3 mice/day of LAB administration. Each assay was performed by duplicate or triplicate.

Microorganisms

The bacterial strains used for experiments were: *L. casei* CRL 431, *L. acidophilus* CRL 924, *Lac. lactis* CRL 526, *L. plantarum* CRL 936, *L. delbrueckii* subsp. *bulgaricus* CRL 423, *L. rhamnosus* CRL 74 and *S. thermophilus* CRL 412, from CERELA culture collection.

L. casei, *L. acidophilus*, *L. rhamnosus*, *L. plantarum* and *L. delbrueckii* ssp. *bulgaricus* were cultured for 8 h at 37°C in MRS (De Man *et al.*, 1960) broth (Oxoid Ltd, U.S.A); *S. thermophilus* and *Lac. lactis* were cultured 8 h at 37°C and 30°C respectively in LAPTg (Lactose, peptone, tryptone-glucose; Raibaud *et al.*, 1961) broth. All of them were harvested by centrifugation at 5,000 g for 10 min, and washed three times with sterile saline solution.

Feeding procedure

Mice were fed daily with each culture of LAB at 10^9 CFU/day/animal for 2, 5 or 7 consecutive days. The cultures, suspended in sterile 10% non-fat milk (NFM) were administered at 20% v/v in the drinking water. The control group received sterile milk (NFM) 10% in the drinking water, given under the same conditions as those used for the test groups.

Immunofluorescence test

The number of IgA-secreting cells, $CD4^+$ and $CD8^+$ T lymphocytes were determined on samples from the ileum near Peyer's patches in the small intestine by immunofluorescence test. The preparation of histological slices of the different groups under study were as described Perdigón *et al.* (1995). The direct immunofluorescence test was performed using the respective monospecific antibodies (α-chain specific) conjugated with fluorescein isothiocyanate (FITC) (Sigma, St. Louis, MO 61378 USA) or FITC conjugates monoclonal antibodies specific for $CD4^+$ or $CD8^+$ T lymphocytes (Gibco BRD Life Technologies, Neutroquimica SA, Buenos Aires 1125, Argentina). Histological samples of small intestine were incubated with 0.2 ml of different antibodies at 1/100 dilutions for IgA or 1/200 for $CD4^+$ and $CD8^+$ during 30 min at room temperature. Then, they were washed three times with 0.01 M Na phosphate-buffered saline, pH = 7.2. Negative controls were run using the respective unlabelled antibodies (α chain specific or $CD4^+$, $CD8^+$) before incubation with FITC-conjugated antibodies. The results were expressed as the mean of number of positive cells *per* 10 fields (magnification 100x). They represent the mean of three histological slices of each animals (n = 3), for each period of administration and LAB.

Histological samples

Histological slices were also stained with haematoxylin-eosin to identify macrophages, eosinophils, polymorphonuclear (PMN) cells and intraepithelial lymphocytes (IEL). The results were expressed as number of cells on 10 villi and *per* 10 fields (magnification 100 x) for IEL. Values represent the mean of three determination from each animal (n = 3) for each period of feeding and LAB.

Histochemical staining

In order to study the mast cells associated with the intestinal epithelium, the histological samples were stained with Alcian-Blue-Safranine at pH = 1.5, according Tass (1977) methodology. The number of mast cells were expressed *per* 10 fields (magnification 100x). Three histological slices from each animals (n = 2) period of administration and LAB, were analized.

Statistical Analysis

Data were expressed as the mean (M) of n independent experiments ± standard errors of the mean (SEM). Student's test was used to calculate the statistical significance of the results.

Results

Determination of IgA producing cells on lamina propria of small intestine

A different pattern in the number of IgA^+ cells through the administration time for each strain was observed. Comparatively to the normal control, we observed that *L. rhamnosus* increased the number of IgA^+ cells with the time of administration. *L. acidophilus* and *L. casei* showed an increase for 2 d of feeding, for 5 or 7 d the values decreased being similar to the normal control. *L. delbrueckii* ssp. *bulgaricus* induced an increase on the IgA cells for all the doses, *S. thermophilus* for 2 and 7 d while *Lac. lactis* showed a slight increase for 5 d and *L. plantarum* enhanced these cells only for 2 d of administration then the value decreased being less than the normal control.

These results are shown in Table 1.

TABLE 1.

Effect of lactic acid bacteria on the IgA secreting cells associated to the small intestine

	IgA secreting cells (Number/10 fields)		
	Days of administration		
Strains	**2**	**5**	**7**
L. rhamnosus	78 ± 5	104* ± 5	112* ± 6
L. acidophilus	131* ± 7	93 ± 6	86 ± 5
L. casei	118* ± 7	68 ± 6	87 ± 4
L. bulgaricus	135* ± 7	102 ± 7	146* ± 6
S. thermophilus	112* ± 5	86 ± 6	120* ± 7
Lac. lactis	93 ± 6	102 ± 5	65 ± 6
L. plantarum	144* ± 7	50 ± 5	40 ± 4

Normal control: 85 ± 5

Histological slices were performed after LAB administration as described in the text. IgA secreting cells were determined by direct Immmunofluorescence test. Values are the mean of n mice = 5 ± SD.* $p < 0.05$

TABLE 2.

Determination of CD4⁺ T cells on lamina propria of small intestine, after LAB administration.

	CD4⁺ T cells (Number/10 fields)		
	Days of administration		
Strains	**2**	**5**	**7**
L. rhamnosus	66 ± 5	24* ± 4	26* ± 5
L. acidophilus	38* ± 4	62 ± 6	65 ± 7
L. casei	131** ± 5	95** ± 5	49 ± 7
L. bulgaricus	31* ± 4	37 ± 5	40 ± 4
Lac. lactis	42 ± 7	62 ± 7	25* ± 4
S. thermophilus	49 ± 6	39* ± 6	39 ± 7
L. plantarum	82** ± 7	29* ± 4	31* ± 5

Normal control: 54 ± 4

LAB were administered for 2, 5 or 7 consecutive days. CD4⁺ T cells were determined at the end of each administration period by Immunofluorescence test on histological slices from small intestine. Results are expressed as mean of n mice= 5 ± SD. *Values significant lower than the control with p between < 0.05 and < 0.01. **Significant higher values than the control with p between < 0.01 and < 0.001.

TABLE 3.

Effect of LAB administration on CD8⁺ T cells number from small intestine

	CD8⁺ T cells (Number/10 fields)		
	Days of administration		
Strains	**2**	**5**	**7**
L. rhamnosus	28* ± 4	22* ± 4	27* ± 5
L. acidophilus	64 ± 6	40 ± 6	39 ± 5
L. casei	50 ± 5	55± 6	49 ± 5
L. bulgaricus	35* ± 7	41± 6	50 ± 7
S. thermophilus	49 ± 5	31*± 5	38* ± 7
Lac. lactis	41 ± 6	54 ± 5	38* ± 5
L. plantarum	107** ± 8	38* ± 7	33* ± 6

Normal control: 58 ± 5

CD8⁺ T cells were counted on lamina propria of histological slices from small intestine by direct Immunofluorescence test. Results are mean of n mice = 5 ± SD. *Values significant lower than the control with $p < 0.01$ and < 0.001. **Significant higher values related to the normal control < 0.01.

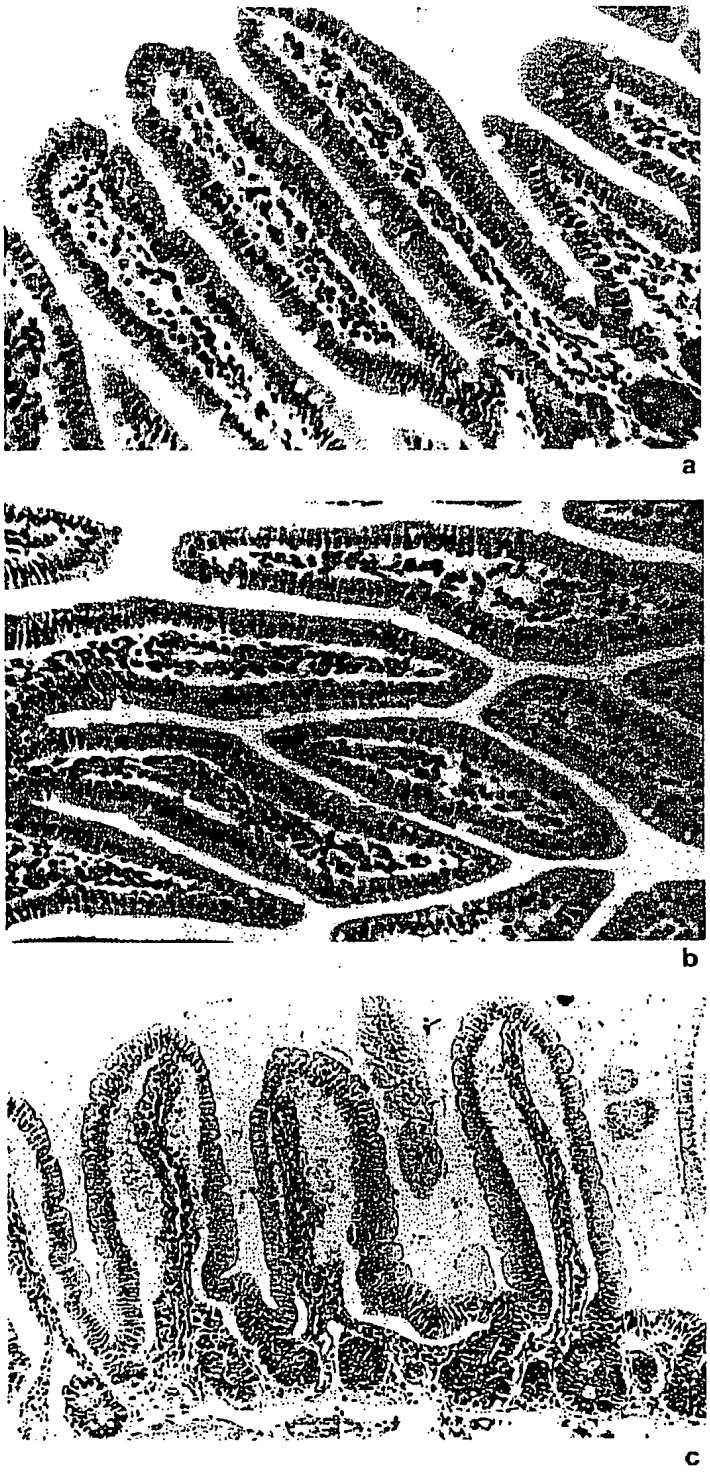

FIGURE 1. Light micrograph of paraffin sections stained with haematoxylin-eosin from small intestine. They show an increase in the number of immune cells associated to the gut (b) or inflammatory response with oedema in the villi (c).
Magnification 40 x.
a) Normal mice; b) Treated with *L. casei* during 2 days; c) Treated with *L. plantarum* during 5 days.

Identification of CD4⁺ and CD8⁺ T cells on lamina propria of small intestine

When we determined CD4⁺ T cells we observed that *L. casei* was able to increase this population for the doses of 2 and 5 d related to the normal control, while *L. plantarum* only did for two days of administration, then the values were lower than the control. *S. thermophilus* and *L. delbrueckii* ssp. *bulgaricus* disminished these T cells for all the doses assayed. *L. rhamnosus*, *L. acidophilus* and *Lac. lactis* did not show an increase in CD4⁺ T cells and the values were lower or similar than the control (see Table 2).

The values obtained for CD8⁺ T cells were lower than the control values in the cases of *L.delbruekii* ssp. *bulgaricus*, *S. thermophilus*, *L. rhamnosus* and *Lac. lactis*. For *L. casei* there was not modification as regard to the control values. *L. acidophilus* showed a not significance increase for 2 d while *L. plantarum* induced a significant increase for 2 d, then they were lower than the control. These results are expressed in Table 3.

Quantification of inflammatory immune cells on lamina propria of small intestine

When we analyzed the number of macrophages present on lamina propria of small intestine we saw an increase of them in relation to the control values for *L. rhamnosus* and *Lac. lactis* for 5 and 7 d of administration. *L. delbruekii* ssp. *bulgaricus*, *S. thermophilus* did not induce augmentation on the macrophage number in the doses assayed. We observed a slight increase for 7 d of *L. casei* and for 2 d of *L. acidophilus*. In the case of *L. plantarum* the values were lower than the control one for 2 and 5 d. There were not modification in the number of polymorphonuclears (PMN) in relation with the

TABLE 4.

Effect of LAB on the immune cells involved in the inflammatory immune response.

Strains	Days of administration	Cells/10 villi		
		Macrophages	PMN	Eosinophils
L.rhamnosus	2	111±5	9 ±2	19*±2
	5	172*±4	21*±3	19*±2
	7	169*±6	13*±2	14±2
Lac.lactis	2	114±4	5±1	13±2
	5	220*±7	26*±2	30*±2
	7	219*±6	28*±3	33*±2
L.acidophilus	2	191*±7	11±2	33*±3
	5	143±6	8±2	30*±2
	7	156±6	16*±2	61*±4
L.casei	2	133±7	9±2	22*±3
	5	110±5	9±2*	19*±3
	7	170*±7	4±1	19*±2
L.bulgaricus	2	91±4	26*±3	20*±2
	5	115±6	16*±2	15±2
	7	100±5	13*±2	3±1
S.thermophilus	2	108±6	22*±2	11±2
	5	119±6	20*±2	4±2
	7	107±6	21*±2	13±2
L.plantarum	2	121±7	23*±3	11±2
	5	106±6	17*±2	7±2
	7	-	-	-

Control Values: Macrophages = 143 ± 6
Polymorphonuclears (PMN) = 6 ± 2
Eosinophils = 10 ± 3

Inflammatory cells were determined on histological slices from small intestine stained with haematoxylin-eosin. Results are mean of n mice = 5 ± SD. *Significant values $p < 0.01$.

control for *L. casei* in all of the dose assayed. *L. acidophilus* increased the number of PMN for 7 d of administration while *L. delbrueckii* ssp. *bulgaricus*, *S. thermophilus*, *L. rhamnosus*, *Lac. lactis* and *L. plantarum* showed PMN cells increase. As regard to the eosinophils number they were increased for *L. acidophilus*, *L. casei*, *L. rhamnosus* and *Lac. lactis* for all the period assayed. *L. delbrueckii* ssp. *bulgaricus* showed an increase only for 2 d. We did not observe eosinophils increase with *S. thermophilus* and *L. plantarum* administration. However for 7 d of *L. plantarum* we could not identify the immune cells due to necrosis. These results are expressed in Table 4. The histological alteration observed when the LAB induced an increase in the number of cells involved inflammatory immune response are showed in Fig. 1 a, b and c. The oedema in the villi is remarkable.

Determination of IEL and mast cell associated with the intestinal epithelium

We observed that most of the LAB assayed increase the number of IEL as regard to the control values. The increase was dose dependent. *L. rhamnosus*, *Lac. Lactis* and *L. casei* shown diminished or similar values to the control. *L. delbrueckii* ssp. *bulgaricus*, *S. thermophilus*, *L. acidophilus* and *L. plantarum* induced an enhance of those cells depending of the dose assayed. When we determined by Alcian-Blue staining if some of the intraepithelial lymphoid cells were mast cells, we observed that *L. rhamnosus* and *L. acidophilus* increased these cells only for 7 days. *Lac. lactis*, *S. thermophilus*, *L. casei* and *L. plantarum* did not increase the mast cells. *L. bulgaricus* showed a significant enhance for 5 and 7 days.

These results are shown in Table 5.

TABLE 5.

Effect of LAB on the number of IEL and Mast cells associated to intestinal epithelium

Strains	Days of administration	Cells/10 fields	
		IEL	Mast cells
L.rhamnosus	2	30.5±3.4	20.22±2.3
	5	53±4.1	19.16±2.8
	7	33.5±2.8	51.17*±2.1
Lac.lactis	2	48.66±3.8	20.83±3.2
	5	58.16*±4.2	30±5
	7	25±4	20.83±3.2
L.acidophilus	2	75.33**±3.2	10.17±3
	5	67.33*±4.3	21.33±2
	7	63.33*±4.1	70.16**±3.1
L.casei	2	38±5.2	21.33±1.7
	5	32.33±6.1	33.1±3.1
	7	37.65±3.1	13±2.4
L.bulgaricus	2	43.33±4.1	12.67±2.1
	5	198.67**±6.2	55.66*±4.1
	7	103.33**±5.1	39.77*±2.3
S.thermophilus	2	60.66*±5.1	18±1.8
	5	38.33±3.3	16.67±5.71
	7	68.33*±6.2	24.16±1.8
L.plantarum	2	61.33*±3.4	19±1.8
	5	131.67**±2.1	17.33±4.3
	7	56.19±3.2	17.1±3.2

Control values: Intraepithelial lymphocytes (IEL) = 45.66 ± 4.1
Mast cells = 20 ± 2

Results are means of n mice = 5 ± SD. Significant values * $p < 0.05$, ** $p < 0.01$

Discussion

The intestinal immune system consists of a number of compartments with different functions contributing to a mucosal immune response. They are: Peyer's patches (PP), gut lamina propria and intra-epithelial lymphocytes (Weinstein and Cebra, 1991; Mc Ghee *et al.*, 1985). Peyer's patches are the inductive site for the generation of IgA committed, preplasmablast and IgA memory cells (Cebra and Shroff, 1994). In this site the antigens are transported through specialized cells called M cells which put in contact the antigen with the antigen presenting cells (APC) to be processed and presented to $CD4^+$ T cells (Walker and Sanderson, 1995). Antigen specific IgA committed B cells and plasmablasts emigrate to the mesenteric lymph nodes, where further expression and maturation of these cells may occur. T cells are also able to migrate (Cerf-Bensussan, 1995). The intestinal lamina propria (LP) receives pre plasma cells from both PP and mesenteric lymph nodes (MLN) (Weiner, 1997), it also receives $CD4^+$ and $CD8^+$ T cells antigen specific or polyclonally induced and non specific NK cells. Antigen specific $CD8^+$ T cells may be generated in PP and emigrate to the intra-epithelial space. In LP are also the immune cells involved in the inflammatory immune response. However, intestinal immunization can induce a profound suppression of the local and also the systemic immune response as just has been reported (Weiner, 1997; Tomasi, 1980). This phenomenon is called oral tolerance and appears to be mediated by cytokines released from immune cells.

In the present paper we studied how the oral administration of LAB interacting with the gut can influence this complexe network of signals between epithelial and immune cells. It was also studied whether or not these microorganisms are able to activate the mucosal immune system and what kind of immune response is induced.

When we analyzed the behaviour of the different LAB assayed on the immune cells associated to mucosa, we saw that each strain showed a different pattern of immune cells activation, especially in the case of IgA producing cells, where this increase, in the most of the LAB studied, was not correlated with an increase in $CD4^+$ T lymphocytes (see Table 1 and 2). It is known the role that plays $CD4^+$ T cell in increasing of IgA^+ B cells favouring the switch IgM to IgA (Weinstein and Cebra, 1991). It was also demonstrated that macrophages and intestinal epithelial cells by antigen stimulation release interleukin 6 (IL6), which can induce IgA^+ cell expression and selective synthesis of this immunoglobulin (Fujihashi *et al.*, 1991). We believe that those LAB that were not able to increase $CD4^+$ T cells, the IgA^+ B cells were enhanced as a consequence of other mechanisms, for example, through IL6 release or by clonal expansion of IgA^+ B cells present in lamina propria. As regard $CD8^+$, with exception of *L. plantarum* cytotoxic T cells were not stimulated. The values were disminished in almost all the LAB assayed (Table 3). This fact, is possitive for the host in order to avoid the cytotoxic immune response. In the case of *L. plantarum*, the necrosis observed for 7 d may be due to an increase in the cytotoxic mechanisms.

When we determined the immune cells involved in the inflammatory immune response we observed an increase in the number of macrophages, neutrophils and eosinophils for the most of the LAB under study (Table 4). These observations would mean an enhance in the inflammatory immune response, which can modify the histological structure of the small intestine (Fig. 1). The increase in the inflammatory cells can also affect the intestinal permeability through the release of substances biologically active as cytokines, produced by cells present in the lamina propria such as PMN, eosinophils or Th1 lymphocytes (Stallmach *et al.*, 1988). Macrophages augmentation would be a possitive fact considering the multiple functions of this cell in the host defense such as phagocytosis of opportunistic, non-pathogenic and pathogenic microorganisms that cross the intestinal barrier to the lamina propria of the gut, where they are phagocyted and killed after phagocytosis (Wells *et al.*, 1988a). We believe that the induction of an inflammatory immune response could favour the uptake of bacteria (translocation of normal microflora) or other antigens present in the intestinal lumen (Wells *et al.*, 1988b). The correlation between inflammatory response and bacterial translocation has been demonstrated in acute inflammation caused by *Shigella* infection (Zychlinsky *et al.*, 1996).

However, it is well demonstrated the role of the mucus layer, which is produced mainly by globet cells after inflammatory stimulus. Mucus is not digested because of its resistance to various enzymes and can protect epithelium against the adherence of pathogens. Mucus secretion can be triggered by direct stimulation of immune complexes and chemical agents and by indirect stimulation by mediator release, such as histamine and lymphokines (Snyder and Walker, 1987).

The effect of various cytokines and growth factors released by immune cells associated to intestinal mucosal, depending of the dose may serve as important modulators of epithelial cell function. Dignass and

Podalsky (1993) demonstrated that some cytokines such us Transforming Growth Factor (TGFβ), cytokines such as IL1β, IFNγ, TNFα and some prostaglandins (PGF) enhanced epithelial cell restitution and they play an important role in sustaining normal mucosal.

The IEL which are $CD8^+$ cells play an important role as protective barrier against infections specially those with T cell receptor (TCR) γ/δ chains (De Libero, 1997). Although we did not determine TCR of IEL some LAB, even when they increased the number of these immune cells associated with the intestine, not all were IEL, we demonstrated the presence of mast cell associated to the gut epithelium. The biological significance of these cells in our experimental model must be determined.

According our results, to assure that certain LAB are able to exert a beneficial effect on the gut, we think that would be important to determine, as a bearing parameter, if the strain assayed is or not able to induce a high inflammatory stimulus to avoid side effects. The inflammatory stimulus could be diminished by decreasing of the daily dose of LAB to be administered, improving the mucosal immunity. As was explained before, a low inflammatory immune response induces beneficial effect in the host. It is also known that an increase in the non specific immune response mediated by PMN would be important by the participation of these cells populations as the first line in the host defense against enteric infections as has been just described (Kagnoff and Eckmann, 1997).

To the light of our results, although the LAB assayed (*L. casei*, *L. acidophilus*, *L. rhamnosus*, *L. plantarum*, *L. delbrueckii* ssp. *bulgaricus*, *S. thermophilus*, *Lac. lactis*) increased the number of IgA immune cells associated to gut, depending of the dose assayed -some of them could induce an enhance in the inflammatory response. The different mucosal immune responses observed, might be due to the different way by which the LAB interact with the small intestine. This last speculation is based in previous reports (Walker and Sanderson, 1995; Weiner, 1997) related to the different ways by which an antigen can interact with the immune cells associated to the gut.

Futher studies for *in vivo* cytokines determination are needed to determine if the $CD4^+$ T cells detected are Th1 or Th2 type, or if the LAB stimulation enhance the release of proinflammatory cytokines such as TNF α or IFN γ, thus increasing the inflammatory response.

We determined the importance in the selection of LAB and the dose to be administered exerting a beneficial effect on the intestine. We also demonstrated that the immunostimulatory capacity of the LAB can not be generalyzed to genus or specie. This property would be restricted to the strain assayed, and not all of the LAB could be use to enhance the intestinal immuntiy.

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Study of the Possible Mechanisms Involved in the Mucosal Immune System Activation by Lactic Acid Bacteria

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ABSTRACT

The induction of a mucosal immune response is not easy due to the development of oral tolerance, but under some conditions, bacteria can activate this immune system. Antigens administered orally can interact with M cells of Peyer's patches or bind to the epithelial cells. We have demonstrated that certain lactic acid bacteria are able to induce specific secretory immunity, and others will enhance the gut inflammatory immune response. The aim of this work was to establish the reason for these different behaviors and to define possible mechanisms involved in the interaction of lactic acid bacteria at the intestinal level. We studied IgA^+ and IgM^+ B cells comparatively in bronchus and intestine and $CD4^+$ T cells and IgA anti-lactic acid bacteria antibodies in the intestinal fluid, induced by oral administration of *Lactobacillus casei*, *Lb. delbrueckii* ssp. *bulgaricus*, *Lb. acidophilus*, *Lb. plantarum*, *Lb. rhamnosus*, *Lactococcus lactis*, and *Streptococcus salivarius* ssp. *thermophilus*. The increase in the IgA^+ B cells in the bronchus means that these lactic acid bacteria were able to induce the IgA cycle by interaction with M cells from Peyer's patches or intestinal epithelial cells. The IgM^+ cells increased when the stimulus did not induce the switch from IgM^+ to IgA^+. The increase in the $CD4^+$ cells suggests interaction of Peyer's patches and enhancement of the B- and T-cell migration. The anti-lactic acid bacteria antibody is related to the processing and presentation of the microorganisms to the immune cells. We demonstrated that *Lb. casei* and *Lb. plantarum* were able to interact with Peyer's patch cells and showed an increase in IgA^+, $CD4^+$ cells, and antibodies specific for the stimulating strain. *Lactobacillus acidophilus* induced gut mucosal activation by interaction with the epithelial cells without increase in the immune cells associated with the bronchus. Although *Lb. rhamnosus* and *Strep. salivarius* ssp. *thermophilus* interact with epithelial cells, they also induced an immune response against their epitopes. *Lactococcus lactis* and *Lb. delbrueckii* ssp. *bulgaricus* induced an increase of IgA^+ cells entering the IgA cycle but not $CD4^+$ cells; thus, these bacteria would have been bound to epithelial cells that activated B lymphocytes without processing and presenting of their epitopes. We did not determine specific antibodies against *Lc. lactis* or *Lb. bulgaricus*.
(**Key words**: lactic acid bacteria, intestinal interactions, immunomodulation)

Abbreviation key: **FITC** = fluorescein isothiocyanate conjugated, **GALT** = gut associated lymphoid tissue, **LAB** = lactic acid bacteria.

INTRODUCTION

The intestinal mucosa is the first line in host defense, and it is exposed to a great number of antigens, many of which are innocuous, while others are potentially harmful. The induction of the immune response at the intestinal level is not always easy. The mechanisms governing immune responsiveness and unresponsiveness in the intestine are not well understood. Orally administered antigens interact with the gut associated lymphoid tissue (**GALT**), which is a well developed immune network that is not only involved in protection of the host from pathogens but also in preventing the host from reacting to ingested protein. Thus, orally administered proteins often induce systemic hyporesponsiveness to the fed proteins; this mechanism is called oral tolerance (6). The maintenance of this process depends on the amount of antigen ingested. Although most dietary antigens are degraded, some intact or partially degraded antigens are absorbed into the systemic circulation. Oral tolerance can be abrogated and an immune response induced (4). This immune response

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is mainly a humoral immune response mediated by IgA producing cells and secretory IgA, which constitute almost 80% of all antibodies produced in mucosal associated tissue (16, 18). These antibodies inhibit the microbial adherence and also prevent absorption of antigens from mucosal surfaces (14). Although all of the immune cells are present on the gut mucosa, the cytotoxic cellular immune response is limited by immunoregulatory mechanisms to avoid intestinal damage (24).

Antigen uptake occurs through the specialized system represented by the M cells overlying Peyer's patches or across normal epithelium overlying the lamina propria (5, 11).

Peyer's patches are the major inductive sites in the intestine in which the antigens have the potential to interact with all of the specialized cells that induce an active immune response between macrophage and dendritic cells (antigen-presenting cells) and T and B lymphocytes. The activation of the oral immune system is clearly associated with activation of T cells in the Peyer's patches. However, many T cells are present in the diffuse lymphoid system of the lamina propria. If the antigen crosses the normal epithelium, it has the potential to present and to activate lamina propria T cells and to induce an immune response.

When the mucosal immune response is induced, primed T and B cells migrate through the lymphatic system and then enter the peripheral blood circulation via the thoracic duct. Extravasation of the immune cells occurs not only in the gut lamina propria but also in other mucosal sites such as respiratory tissues, urogenital tissues, and mammary and salivary glands. This migration is known as the IgA cycle (23), and its distribution from the gut to other mucosal sites has been described as the common system of mucosa (17). This concept implies that oral immune stimulation can induce immunity in mucosal sites remote from the gut.

Much relevant literature (2, 7, 9) has demonstrated the importance both to human and animal health of the lactic acid bacteria (LAB) present in fermented foods and probiotic preparations. Some LAB can affect protective immunity against pathogens and tumors and have the ability to increase the mucosal immune response (9, 10, 13, 15).

In our laboratory, we have demonstrated the capacity of certain LAB to enhance both systemic and mucosal immunity. Furthermore, we have shown that dose can influence the effect achieved (1, 21, 22, 27).

Although much research has been done, the use of LAB for therapeutic purposes is still limited. The effect of LAB may be influenced by differences between strains, model systems, or poor viability. Thus, it is very difficult to generalize from results of one experiment.

Knowledge of the possible mechanisms by which LAB interact at the intestinal level and then stimulate the immune cells could improve the use of these microorganisms as immunomodulator substances.

The aim of this research was to study the effect of LAB on immune cells associated with the mucosa and other immune parameters to determine the possible site of interaction of these microorganisms with the small intestine. This knowledge could explain why not all of the LAB could stimulate the immune system in the same way.

MATERIAL AND METHODS

Microorganisms Used

The bacterial strains used for the experiments were *Lb. casei* CRL 431, *Lb. acidophilus* CRL 924, *Lb. plantarum* CRL 936, *Lb. delbrueckii* ssp. *bulgaricus* CRL 423, *Lb. rhamnosus* CRL 74, *Lc. lactis* CRL 526, and *Strep. salivarius* ssp. *thermophilus* CRL 412 from the Centro de Referencias para Lactobacilos (CERELA, Tucumán, Argentina) culture collection. *Lactobacillus casei*, *Lb. acidophilus*, *Lb. rhamnosus*, and *Lb. plantarum* were cultured for 8 h at 37°C in MRS broth (Oxoid Ltd., Hampshire, England); *Strep. thermophilus* and *Lc. lactis* were cultured 8 h at 37°C and 30°C, respectively, in LAPTg broth. All bacteria were harvested by centrifugation at 5000 × *g* for 10 min and were washed three times with sterile saline solution.

Animals and Feeding Procedure

The BALB/c mice weighing 25 to 30 g were obtained from the random-bred colony kept in our department at the Institute of Microbiology. Each experimental group consisted of 5 to 6 mice.

Each culture of LAB was suspended in 5 ml of sterile nonfat milk and was administered at 20% (vol/vol) in the drinking water to different groups of mice for 2, 5, or 7 consecutive d at a dose of 10^9 cells/d per mouse. The control group received sterile milk in the drinking water given under the same conditions as for the test group.

All of the mice were fed ad libitum with a conventional balanced diet.

Tissue Sections

At the end of each feeding period with the different LAB, mice were anesthetized and slaughtered, and

the small intestine and the lower respiratory tract were removed. Tissues were placed in ethanol and were processed by the technique of Saint Marie (25). Tissues were fixed, dehydrated, and embedded in paraffin at 56°C. From each tissue, 4-μm serial paraffin sections were cut.

Immunofluorescence Test

The IgA, IgM-secreting cells, and CD4$^+$ lymphocytes were determined on histological slices from the gut and bronchus.

Direct immunofluorescence was performed using the respective monospecific antibodies (α- or μ-chain specific) conjugated with fluorescein isothiocyanate (FITC) (Sigma Chemical Co., St. Louis, MO) or FITC-conjugated monoclonal antibodies specific for CD4$^+$ lymphocytes (Gibco BRD Life Technologies, Neutroquimica, Argentina).

Negative controls were run using the respective unlabelled antibodies before incubation with FITC-conjugated antibodies.

Results were expressed as the mean of the number of positive cells per 10 fields of light microscope (100×).

Anti-LAB Antibodies and ELISA Test

The anti-LAB antibodies present in intestinal fluid were measured by ELISA. Assays were performed at the end of each feeding period and for each LAB that was assayed.

The procedures used for collection of intestinal fluid and ELISA were as described previously by Alvarez et al. (1) and used goat anti-mouse IgA (α-chain specific conjugated peroxidase; Sigma Chemical Co.). Absorbance was measured at 493 nm.

Control values were those obtained from intestinal fluid of untreated mice for each LAB under study.

Statistical Analysis

The number of different immune cells measured were determined as arithmetic means of the values obtained from (n = 5 to 6) determinations expressed for each surface marker. Statistical comparisons were conducted using Student's test.

RESULTS

Determination of the Number of IgM$^+$ Cells in GALT

The number of IgM$^+$ B cells was measured because their importance is second only to that of IgA in protection of mucosal surfaces.

We observed that only *Lc. lactis*, *Lb. acidophilus*, and *Lb. plantarum* were able to increase the number of IgM-secreting cells in the lamina propria of the small intestine for different periods of administration: 2, 5, and 7 d, respectively (see Figure 1).

Study of IgA Producing Cells on GALT

Oral administration of all LAB strains studied increased the number of IgA cells in the lamina propria. The effect was dose dependent. The most remarkable effect was obtained with *Lb. casei*, *Lb. plantarum*, *Lb. delbrueckii* ssp. *bulgaricus*, and *Strep. salivarius* ssp. *thermophilus* (see Figure 2). We also observed a significant decrease ($P < 0.01$) when *Lb. plantarum* was administered for 5 d.

Effect of LAB on CD4$^+$T Cells

The CD4$^+$ T cells are important in the induction of specific immune responses, and they participate in the switch from IgM to IgA.

We demonstrated that only *Lb. casei* and *Lb. plantarum* were able to increase CD4$^+$ T cells in the lamina propria of the small intestine. The effect was dose dependent.

The values obtained with other LAB were similar to those of controls with exceptions for *Lb. rhamnosus* at 2, 5, and 7 d and for *Lc. lactis* at 7 d, for which we observed a significant decrease ($P < 0.01$) in the number of CD4$^+$ cells. These results are in Figure 3.

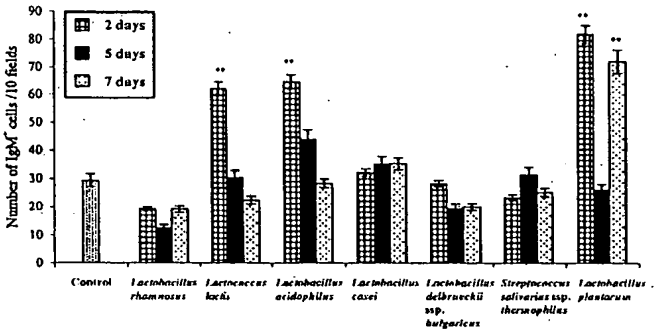


Figure 1. Effect of oral administration of different LAB on the number of IgM cells present in lamina propria of the small intestine tissue. The IgM B cells were measured by an immunofluorescence test using μ-chain monospecific antibody after 2, 5, or 7 d of feeding with each lactic acid bacteria assayed. The bars indicate arithmetic means (±SD) of the number of cells in 10 fields (100×) expressing the marker. $^{**}P < 0.01$.

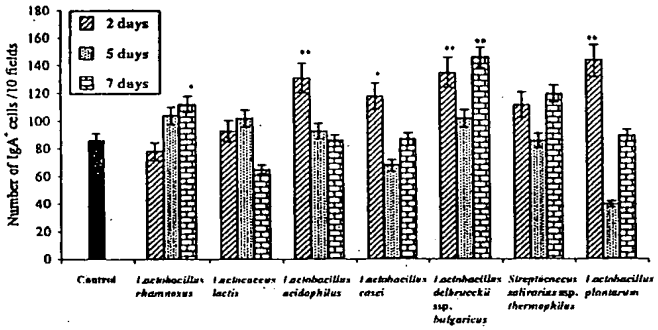


Figure 2. Effect of oral administration of different lactic acid bacteria on the number of IgA+ B cells. They were measured by an immunofluorescence test using monospecific antibody after 2, 5, or 7 d of feeding with each lactic acid bacteria assayed. The bars indicate arithmetic means (±SD) of the number of cells in 10 fields (100×) expressing the marker. $^{*}P < 0.05$. $^{**}P < 0.01$.

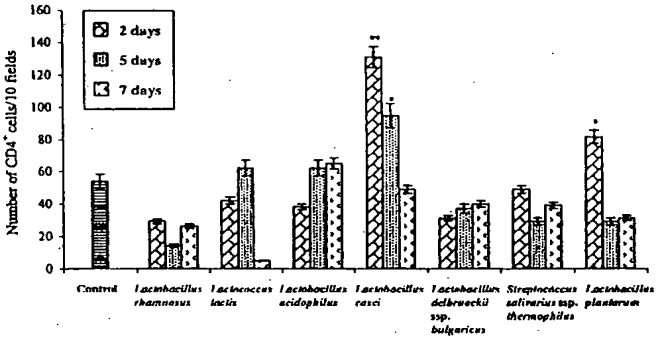


Figure 3. Effect of oral administration of different lactic acid bacteria on the number of CD4+ T cells. They were measured by an immunofluorescence test using CD4 monoclonal antibody after 2, 5, or 7 d of feeding with each lactic acid bacteria assayed. The bars indicate arithmetic means (±SD) of the number of cells in 10 fields (100×) expressing the marker. $^{*}P < 0.05$. $^{**}P < 0.01$.

Determination of the IgA B Cells Associated with Bronchus Tissue

We studied the IgA-secreting cells associated with bronchus associated lymphoid tissue as a measure of cellular migration. Oral antigen stimulation can induce IgA B cells present in the gut to enter to the IgA cycle and to increase the number of these cells in sites distant from the intestine (such as bronchus) under the concept of common system of mucosa.

We observed that all of the LAB assayed, with the exception of *Lb. acidophilus*, were able to increase the number of IgA+ cells in bronchus. This effect was dose dependent. These results are shown in Figure 4.

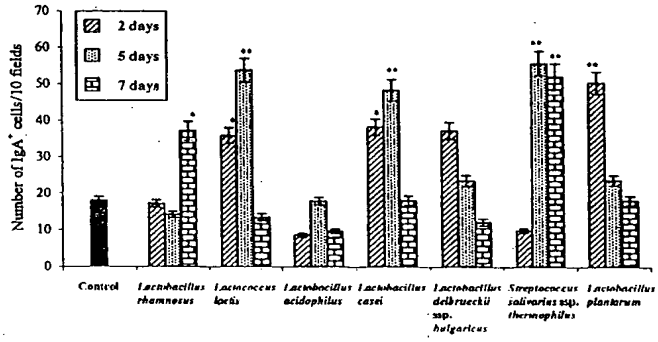
Study of the Anti-LAB Antibodies

This assay was performed to determine whether the LAB as antigens were degraded and presented to the immune cells to induce antibody production against their epitopes. Antigen degradation may be in the Peyer's patches or in the epithelial cell. We determined that only *Lb. casei*, *Lb. rhamnosus*, *Strep. salivarius* ssp. *thermophilus*, and *Lb. plantarum* were able to induce antibody production (see Table 1).

DISCUSSION

In the induction of the gut immune response, the increase in the number of IgA producing cells is due to the switch of IgM+ B cells present in Peyer's patches to IgA+ B cells under the influence of cytokines released by CD4+ T-helper lymphocytes type 2 (Th2) (12, 19). An increase in the number of IgM+ cells in lamina propria would mean that the stimulus was only able to mobilize these B lymphocytes without activation of Th2 lymphocytes. Only stimulation by *Lc. lactis* (2 d), *Lb. acidophilus* (2 and 5 d), and *Lb. plantarum* (2 and 7 d) (Figure 1) led to an increase in the number of IgM+ cells in the lamina propria.

To have good stimulation of intestinal mucosa, the number of IgA+ B lymphocytes must be only slightly higher than in the normal control (3). High numbers of IgA+ cells could have harmful effects on the host and favor an increase in intestinal permeability, which occurs with coeliac disease (26). When we determined the number of IgA+ cells present in the small intestine, we saw different patterns between the LAB assayed for the different periods of adminis-



bacteria on the number of IgA cells associated the bronchus associated lymphoid tissue. IgA+ B cells were measured by an immunofluorescence test using monospecific antibody after 2, 5, or 7 d of feeding with each lactic acid bacteria assayed. The bars indicate arithmetic means (±SD) of the number of cells in 10 fields (100×) expressing the marker. $^{*}P < 0.05$. $^{**}P < 0.01$.

TABLE 1. Determination of antibodies for anti-lactic acid bacteria (LAB) by ELISA.[1]

Microorganisms	Control		LAB administration (absorbance at 493 nm)					
			2 d		5 d		7 d	
	$\bar{X}$	SD	$\bar{X}$	SD	$\bar{X}$	SD	$\bar{X}$	SD
Lactobacillus casei	0.083	0.042	0.274**	0.022	0.342**	0.053	0.352**	0.028
Lactobacillus rhamnosus	0.167	0.031	0.226	0.057	0.476**	0.019	0.362**	0.025
Streptococcus salivarius ssp. *thermophilus*	0.163	0.027	0.285*	0.035	0.252*	0.010	0.132	0.051
Lactobacillus delbrueckii ssp. *bulgaricus*	0.124	0.018	0.143	0.030	0.147	0.051	0.151	0.042
Lactobacillus acidophilus	0.382	0.048	0.369	0.063	0.402	0.055	0.394	0.065
Lactobacillus plantarum	0.148	0.010	0.280**	0.032	0.193	0.060	0.207*	0.028
Lactococcus lactis	0.095	0.018	0.156	0.033	0.150	0.040	0.086	0.037

[1]The mice (n = 5) were fed with different LAB for 2, 5, or 7 consecutive d. At the end of each feeding period, they were slaughtered, and the intestinal fluid was recovered to perform an ELISA test. Control mice were without treatment.

*$P < 0.05$.

**$P < 0.01$.

tration (Figure 2), which suggested that the dose administered could affect the results. The great decrease in the IgA$^+$ cells observed for d 5 of *Lb. plantarum* may be due to a down regulation process to avoid an increase in the inflammatory immune response. When we analyzed the number of IgA$^+$ cells on bronchus tissue, we observed that *Lb. casei*, *Lb. plantarum*, *Strep. salivarius* ssp. *thermophilus*, *Lb. rhamnosus*, *Lb. delbrueckii* ssp. *bulgaricus*, and *Lc. lactis* were able to increase the number of IgA$^+$ B lymphocytes in bronchus (Figure 4). This effect was also dose dependent. The increase in IgA$^+$ cells in GALT and bronchus associated lymphoid tissue suggest that cellular migration was taking place.

Antigen-presenting cells from the Peyer's patches or lamina propria of the small intestine may induce an antigen specific proliferative response of T cells (20). However, T cells and IgA$^+$ B cells can leave the Peyer's patches following antigen stimulation and increase the number of T cells in the lamina propria. If the antigen interaction is with epithelial cells, CD4$^+$ T cells can be activated to stimulate other immune cells through the release of cytokines, but they are not able to migrate to sites distant from the intestine (20). Clear differences were noted in the profile of cytokines when T cells were stimulated in lamina propria. The presentation of antigen by cells from the lamina propria would lead to a lack of conventional activation and differentiation (29).

When we studied the number of CD4$^+$ T cells, we observed that most of the LAB assayed were not able to increase this population with exception of *Lb. casei* and *Lb. plantarum* for some periods of administration. On the contrary, the most frequent effect observed was a decrease in the number of these cells especially for *Lb. rhamnosus* and *Lc. lactis* (Figure 3). We do not think that these LAB are able to induce selectively a suppression of immune response. We believe that the decrease observed could be related to the interaction of these LAB with the intestinal epithelial cells and to the levels of cytokine released, which would not be enough to induce clonal expansion of the T cells present in lamina propria. The CD4$^+$ increase in the lamina propria means not only cellular migration but also interaction of LAB with M cells of the Peyer's patches. This fact would mean that only *Lb. casei* and *Lb. plantarum* could induce an effective stimulation from Peyer's patches of the immune cells associated with the gut. The diminution of these cells might also be due to the mechanisms of oral tolerance, which can be mediated by deletion, anergy, or active cellular suppression for which the determining factor is the dose of antigen fed. Low doses favor active suppression, whereas high doses favor deletion and anergy (28).

When we determined whether the LAB were processed as antigens and whether they induced an immune response against their own epitopes, we detected antibodies against LAB for *Lb. casei*, *Lb. plantarum*, *Lb. rhamnosus*, and *Strep. salivarius* ssp. *thermophilus*, which indicated that these LAB were degraded and presented to the immune cells (Table 1).

We postulated the following hypothesis, taking into account results obtained for number of IgA+ and CD4+ cells and antibodies against LAB, about the way in which the different LAB can interact with the small intestine. If LAB induce an increase in the number of IgA$^+$ cells in gut and bronchus, on the CD4$^+$ T cells, and in the immune response against their epitopes, the interaction could have been through M cells at the Peyer's patches such as was

observed using *Lb. casei* and *Lb. plantarum*. When we observed increases in IgA$^+$ cells in the gut and bronchus, but no increase in the CD4$^+$ T cells, the interaction would have been on the intestinal epithelium cells independent of whether or not the LAB were processed as antigen. The IgA cycle may also be increased by mobilization of the IgA$^+$ cells present in the mesenteric lymph node (28), which we observed with *Lb. rhamnosus* and *Strep. salivarius* ssp. *thermophilus*, which were processed and presented as antigen. *Lactobacillus delbrueckii* ssp. *bulgaricus* and *Lc. lactis* could interact at epithelial level without processing these microorganisms but increasing the IgA cycle.

We believe that *Lb. acidophilus* has only a local effect on the gut when interacting with the epithelial cells. It was unable to induce the IgA$^+$ cycle or to increase the number of CD4$^+$ T cells. The effect induced would be exerted only by the cytokines released by the stimulation of the epithelial cells.

In this report we demonstrated that LAB can interact at different levels in the small intestine. These interactions could explain why the mucosal immunostimulation by LAB is not the same for all bacterial LAB species.

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Intestinal Immune Response to Oral Administration of *Lactobacillus reuteri* R2LC, *Lactobacillus plantarum* DSM 9843, Pectin and Oatbase on Methotrexate-induced Enterocolitis in Rats

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Dietary administration of lactobacilli and certain fibres significantly reduced the severity of methotrexate-induced enterocolitis in rats by improving intestinal mucosal structure, reversing microbiota disruption, possibly also influencing intestinal mucosal immunity. The purpose of this study was to evaluate the effects of two *Lactobacillus* strains and fibres, which have been proven to be effective in enterocolitis of rats, on gut immune response. All rats received continuous intragastric infusion of an elemental diet with or without supplementation of pectin, oatbase, and *Lactobacillus reuteri* R2LC or *Lactobacillus plantarum* DSM 9843 from the beginning of the study via a gastrostomy. The control group rats had normal chow throughout the study. On day three, animals received intraperitoneal injections of either methotrexate, 20 mg/kg, or normal saline, and the sampling was done on day six. Administration of methotrexate significantly diminished intestinal secretory IgA level and gut *lamina propria* CD4/CD8 lymphocyte counts. The addition of *L. reuteri* R2LC, and *L. plantarum* DSM 9843, but not of pectin and oatbase, significantly increased the ileal and colonic secretory IgA level, both in soluble and insoluble fractions, and elevated CD4 and CD8 numbers compared with control enterocolitis rats. The enhancement of gut immune function by lactobacilli administration may be one of the important processes through which certain strains of lactobacilli facilitated the recovery from enterocolitis.

KEY WORDS: intestinal immunity; enterocolitis; *Lactobacillus reuteri*; *Lactobacillus plantarum*; pectin; oat.

INTRODUCTION

The gastrointestinal tract, traditionally viewed as an organ of digestion and absorption of nutrients, has been recognised as one of the largest immune organs in the body, which not only provides indispensable immune function against its own resident microbiota, but in addition provides significant effective immune function to distant sites such as the liver, breast, lung etc.[3] It is known that the intestinal immune response plays a crucial role in protecting the host from invasion of pathogens.[22] Central to it is the production and expression of secretory IgA (sIgA), the most abundant immunoglobulin in external secretions. Data from humans and animals clearly demonstrate an association between IgA deficiency and impairment of intestinal barrier function.[1,18] In certain circumstances, such as severe damage of intestinal mucosa, improper nourishment of the gut and significant alteration of the intestinal microecology, the gut immune system may be partially destroyed.[1]

In recent years, increased emphasis has been placed on the study of the therapeutic effects of lactobacilli on various human and animal gastrointestinal disorders.[21] It is known that the presence of lactobacilli in fermented food products is important not only because of their nutritional value, but also because certain strains of lactobacilli have immunopotentiating properties.[25] Perdigon and Sato have shown that administration of certain strains of *L. casei* and *L. acidophilus* augmented cellular functions of both liver and peritoneal macrophages,[21,25] and increased the sIgA production secreted into the intestinal lumen.[22]

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Previous studies[9,15] have indicated that administration of methotrexate (MTX) to rats induces a severe enterocolitis with bacteraemia and endotoxaemia. It has also been demonstrated that dietary supplementation of certain fibres, such as pectin and oatbase, or *L. plantarum* and *L. reuteri* species dramatically attenuated the intestinal injury, decreased the bacteraemia and endotoxaemia. Possible mechanisms include improvement of intestinal mucosal structure, physical barrier function and reversal of the intestinal microbiota disruption.[15,16] However, it is not known if the intestinal immune response is also involved in the mechanisms by which MTX adversely affects the host animals, and the two tested *Lactobacillus* strains and fibres facilitate the recovery of the enterocolitis.

The purpose of the present study was to examine the response of luminal sIgA level and *lamina propria* T-helper/T-suppressor lymphocyte counts, both in the small bowel and colon, to MTX injection, and oral administration of fibre free elemental diet, pectin, oatbase, *L. reuteri* R2LC, and *L. plantarum* DSM 9843 in rats with MTX-induced enterocolitis.

MATERIALS AND METHODS

Chemicals

Fibre free elemental diet (ED) was purchased from Nutricia Nordica AB (Solna, Sweden); methotrexate sodium salt was from Lederle Arzneimittel (Wolfratshausen, Germany); citrus pectin, peroxidase conjugated anti-mouse IgG and anti-goat IgG were obtained from Sigma Chemical Co. (St Louis, MO, USA); Goat anti-rat sIgA [H ch+sec pc] was purchased from ICN (Costa Mesa, CA, USA), and monoclonal antibodies to T helper cells and T suppressor cells from Cedarlane Lab Ltd (Ontario, Canada); Oatbase and *L. reuteri* R2LC, *L. plantarum* DSM 9843 in fermented oatbase were prepared and provided by the Department of Food Technology, Lund University.

Animals

Forty-two male, Sprague-Dawley rats (Möllegård, Viby, Denmark), weighing 200–250 g were housed individually in wire-bottomed cages to limit coprophagy. They were acclimatised for 5 d at a constant temperature with 12 h periods of dark and light cycles and allowed *ad libitum* intake of standard rat chow (R3, Lactamin AB, Stockholm) and water. Three days before experiment, all the animals were fed ED *ad libitum* in order to eliminate the effect of residual fibre on intestinal nutrition. On the day of experiment (day 0), 36 rats were weighed, and had placement of a gastrostomy under ether anaesthesia fixed with a swivel apparatus to allow long-term infusion. The animals received intragastric infusions (2·0 ml/h) of normal saline overnight postoperatively and were randomised into six different groups with different diets on day 1 (Table 1). The remaining six rats had sham operations (insertion of a gastrostomy tube with other end blocked under the skin) and free access to food (Chow group). All animals received the assigned diet in isocaloric and isonitrogenous amounts. The experimental design was approved by the Animal Ethics Committee of Lund University and adhered to the Guiding Principles in the Care and Use of Animals.

Harvesting of specimens

Rats were sacrificed by an overdose of ether, and a midline incision was made. The small intestine from the ligament of Treitz to the ileocecal valve and whole colon were excised and freed of their mesenteric fat. The small intestine was then divided in half (into jejunum and ileum). The contents of excised ileum and colon were washed out (containing soluble fraction of sIgA) with 5 ml of chilled phosphate-buffered saline (PBS, pH 7·2) according to the method of Spaeth *et al.*[27] with modification. The contents were then centrifuged at 3000 *g* for 20 min at 4°C, the supernatants were filtered using 0·2 μm filters (Sartorius Filtration AB, Sundbyberg, Sweden), and stored at −70°C for sIgA assay. The ileum and colon were opened longitudinally and the mucosa including the mucous gel were scraped off (containing insoluble fraction of sIgA) with a glass slide and homogenised with 5 ml chilled PBS in a blender at 30 000 r.p.m. for 30 s. The homogenates were centrifuged at 3000 *g* for 20 min. After the filtration as above, the supernatants were stored at −70°C for sIgA measurement. The lengths of the ileum and colon were recorded under a fixed tension.

Portions of distal ileum and colon were excised for determination of gut *lamina propria* T-helper (CD4) and T-suppressor (CD8) lymphocytes.

Histopathological study

Five millimeter samples of distal ileum and proximal colon were obtained and placed in

Table 1. Experimental design

<table>
<tr><th rowspan="2">Groups</th><th colspan="5">Experimental procedure</th></tr>
<tr><th>Day 0</th><th>Day 1–2</th><th>Day 3</th><th>Day 4–5</th><th>Day 6</th></tr>
<tr><td>1. Chow (n=6)</td><td>Sham operation,
Free access to chow and water postoperatively</td><td>Free access to rat chow and water</td><td rowspan="2">i.p. injection of 2 ml normal saline</td><td rowspan="7">Same diet or infusion as day 1–2</td><td rowspan="7">Sacrificed for sampling</td></tr>
<tr><td>2. ED (n=6)</td><td rowspan="6">Gastrostomy, intragastric infusion of normal saline over night postoperatively (2·0 ml/h)</td><td>Continuous intragastric infusion (2·5 ml/h) of ED base (240 kcal/kg and 1·28 g nitrogen/kg/day), plus additional 2·1 ml ED/rat/day for intra-groups energy balance</td></tr>
<tr><td>3. MTX (n=6)</td><td>Same intragastric infusion as group 2</td><td rowspan="5">i.p. injection of MTX, 20 mg/kg</td></tr>
<tr><td>4. Pectin (n=6)</td><td>Same intragastric infusion as group 2, with supplementation of 1% Pectin</td></tr>
<tr><td>5. Oat (n=6)</td><td>Continuous intragastric infusion of ED base as group 2, plus 4 ml oatbase/rat/day</td></tr>
<tr><td>6. R2LC (n=6)</td><td>Continuous intragastric infusion of ED base as group 2, plus 4 ml of L. reuteri R2LC in fermented oatbase/rat/day</td></tr>
<tr><td>7. DSM (n=6)</td><td>Continuous intragastric infusion of ED base as group 2, plus 4 ml of L. plantarum DSM 9843 in fermented oatbase/rat/day</td></tr>
</table>

ED, elemental diet; i.p. injection, intraperitoneal injection; MTX, methotrexate.

phosphate buffered 4 per cent formadehyde. Paraffin-embedded samples were cut and studied under light microscopy after staining with haematoxylin and eosin. At least three sections were studied from each specimen in a blind fashion.

Intestinal sIgA assay

SIgA was measured using the quantitative enzyme-linked immunosorbent assay (ELISA),[10] with purified rat bile sIgA[12] as a standard, and goat anti-rat sIgA as a primary antibody. The

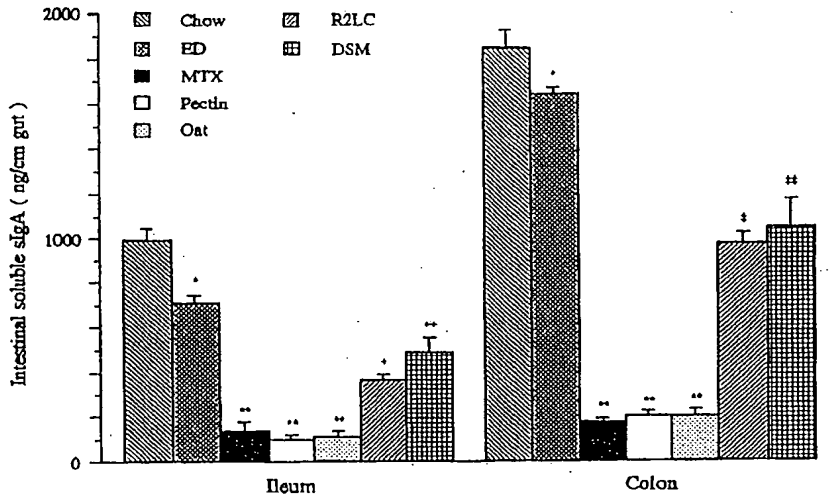

Figure 1. Different levels of soluble fraction of secretory IgA in ileal and colonic washing from all groups. Samples were taken 72 h after intraperitoneal (i.p.) injections of either methotrexate (MTX, 20 mg/kg) or saline. Values are expressed as means ± SE. Chow and ED, groups of rats fed either chow or elemental diet, had i.p. injection of saline. MTX, a group of rats fed elemental diet, had i.p. injection of MTX. Pectin, Oat, R2LC, DSM, groups of rats fed elemental diet with supplementation of pectin, oatmeal, *L. reuteri* R2LC, and *L. plantarum* DSM 9843, and had i.p. injection of MTX. $^{*}P<0{\cdot}05$ *vs.* Chow. $^{**}P<0{\cdot}001$ *vs.* Chow and ED. $^{+}P<0{\cdot}001$ *vs.* Chow, ED, Pectin, and Oat; $P<0.01$ *vs.* MTX. $^{++}P<0{\cdot}01$ *vs.* Chow, MTX, and Pectin; $P<0{\cdot}05$ *vs.* ED; $P<0{\cdot}001$ *vs.* Oat. $\ddagger P<0{\cdot}001$ *vs.* Chow, ED, MTX, Pectin, and Oat. $\ddagger\ddagger P<0{\cdot}01$ *vs.* Chow, ED, and Pectin; $P<0{\cdot}001$ *vs.* MTX and Oat

reaction was developed with further incubation of peroxidase conjugated anti-goat IgG. Absorbance was measured at 450 nm. The soluble sIgA fraction was measured in the intestinal washing and the insoluble fraction was determined in the mucosal homogenate. Both fractions were expressed per length of the respective intestinal segment.

Immunoperoxidase staining and counting of gut lamina propria *lymphocytes*

The sections of distal ileum and proximal colon were assessed by the direct immunoperoxidase technique to prevent excessive background staining.[8] These sections were deparaffinised by xylene treatment and progressive rehydration in decreasing concentrations of alcohol (100–96 per cent). Tissue sections were immersed in a hydrogen peroxide and methanol mixture, to block endogenous peroxidase activity, for 10 min.

Anti-rat T helper cell and T suppressor cell monoclonal antibodies (1:1000) were used and each slide was coated, incubated for 1 h, and rinsed with PBS for 15 min to eliminate unbound antibody. These slides were further incubated with peroxidase, conjugated anti-mouse IgG for 30 min and treated with a substrate solution for 5 min. The numbers of CD4 and CD8 lymphocytes in the *Lamina propria* were counted under a light microscope. At least five fields were reviewed per slide.

Statistical analysis

Data are presented as the mean ± SE. Secretory IgA levels and CD4, CD8 lymphocyte counts between groups were analysed using Student's *t*-test. Statistical significance was considered when P was less than 0·05.

RESULTS

Rats in all treatment groups appeared significantly less affected by diarrhoea and lethargy compared with those in the MTX group. All animals, except Chow group rats (12·5 ± 0·4 per cent body weight increase) lost weight during the study period. The

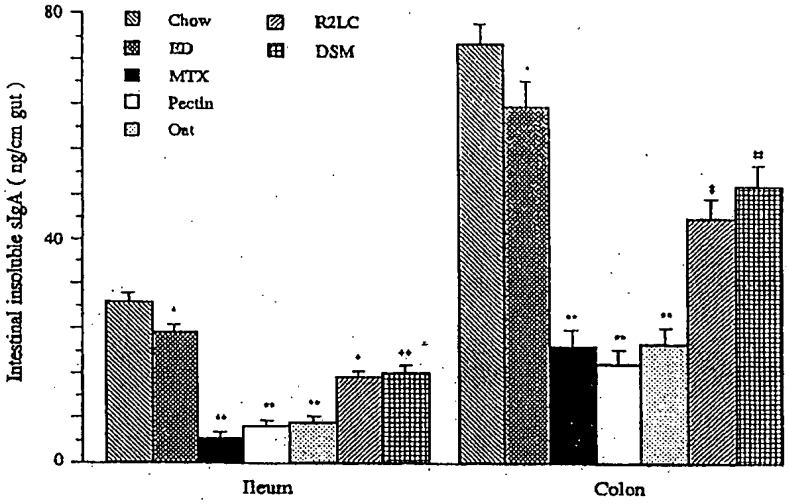

Figure 2. Different levels of insoluble fraction of secretory IgA in ileal and colonic mucosa homogenate from all groups. Samples were taken 72 h after i.p. injections of either MTX (20 mg/kg) or saline. Values are expressed as means ± SE. Chow and ED, groups of rats fed either chow or elemental diet, had i.p. injection of saline. MTX, a group of rats fed elemental diet, had i.p. injection of MTX. Pectin, Oat, R2LC, DSM, groups of rats fed elemental diet with supplementation of pectin, oatmeal, *L. reuteri* R2LC, and *L. plantarum* DSM 9843, and had i.p. injection of MTX. $^{*}P<0{\cdot}05$ *vs.* Chow. $^{**}P<0{\cdot}001$ *vs.* Chow and ED. $^{+}P<0{\cdot}001$ *vs.* Chow and Oat; $P<0.01$ *vs.* ED, MTX, and Pectin. $^{++}P<0{\cdot}01$ *vs.* Chow and Oat; $P<0{\cdot}05$ *vs.* ED; $P<0{\cdot}001$ *vs.* MTX and Pectin. $\ddagger P<0{\cdot}01$ *vs.* Chow, MTX, and Oat. $P<0{\cdot}05$ *vs.* ED; $P<0{\cdot}001$ *vs.* Pectin. $\ddagger\ddagger P<0{\cdot}01$ *vs.* Chow; $P<0{\cdot}05$ *vs.* ED; $P<0{\cdot}001$ *vs.* MTX, Pectin, and Oat

two *Lactobacillus* treated groups (R2LC, 3·2 ± 0·3 per cent decrease; DSM, 3·1 ± 0·6 per cent decrease) and the ED group (3·0 ± 0·1 per cent decrease) lost significantly less body weight and had better general conditions compared with the Pectin group (6·2 ± 0·4 per cent decrease) and Oat group (7·4 ± 0·5 per cent decrease). MTX group rats lost the largest percentage of their body weight (11·1 ± 0·5 per cent decrease) among all the experimental groups.

On gross examination, the excised small and large intestines disclosed severe diffuse injury indicated by oedema and congestion without perforation in MTX rats, and much milder changes in treatment group rats. Under microscopic examination, MTX rats exhibited a significant loss of the mucosal villous tips along with severe inflammation and ulceration. In the enterocolitis rats treated with pectin, oatbase and the two strains of *Lactobacillus*, the mucosal histology was much improved. Intestinal samples from the Chow and ED groups had a normal and a slightly atrophic mucosal histology, respectively.

Intestinal concentrations of soluble and insoluble sIgA between the study groups are summarised in Figures 1 and 2. Animals administered MTX without other treatment had a significant decrease in soluble and insoluble sIgA both in the ileum and the colon compared with the Chow and ED group. The addition of oatbase and pectin to enterocolitis rats did not significantly alter the sIgA levels in the ileum or colon, whereas supplementation of the two strains of lactobacilli to enterocolitis rats significantly increased the soluble and insoluble sIgA levels in both ileum and colon. The sIgA levels from the two *Lactobacillus* treated enterocolitis groups were, however, still lower than those of control rats in Chow and ED groups. No statistically significant difference in sIgA level was found between the two *Lactobacillus* treatment groups (R2LC *vs.* DSM), or between the two fibre groups (Pectin *vs.* Oat). The animals fed ED in this study had significantly lower values in soluble and insoluble sIgA in the ileum and colon compared with the animals fed normal rat chow.

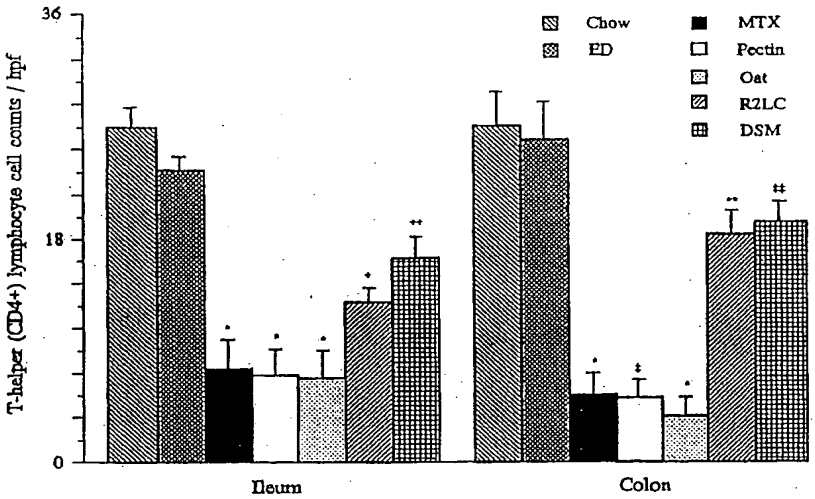

Figure 3. Different T-helper (CD4+) lymphocyte cell counts in the intestinal *lamina propria* from different dietary group rats received i.p. injections of either MTX (20 mg/kg) or saline. Samples were taken 72 h after the injections. Values are expressed as means ± SE. Chow and ED, groups of rats fed either chow or elemental diet, had i.p. injection of saline. MTX, a group of rats fed elemental diet, had i.p. injection of MTX. Pectin, Oat, R2LC, DSM, groups of rats fed elemental diet with supplementation of pectin, oatmeal, *L. reuteri* R2LC, and *L. plantarum* DSM 9843, and had i.p. injection of MTX, hpf, high power field. *$P<0.001$ *vs.* Chow and ED. **$P<0.05$ *vs.* Chow and ED; $P<0.001$ *vs.* MTX, Pectin and Oat. $^{+}P<0.001$ *vs.* Chow and ED; $P<0.05$ *vs.* MTX, Pectin, and Oat. $^{++}P<0.01$ *vs.* Chow and ED; $P<0.05$ *vs.* MTX, Pectin, and Oat. ‡$P<0.001$ *vs.* Chow; $P<0.01$ *vs.* ED. ‡‡$P<0.05$ *vs.* Chow and ED; $P<0.01$ *vs.* MTX and Pectin; $P<0.001$ *vs.* Oat

Data from gut *lamina propria* T-helper/T-suppressor cell (CD4/CD8) populations are summarised in Figure 3 and Figure 4. Animals injected with MTX showed significant decreases in CD4 and CD8 populations from both ileum and colon. Administration of both *L. reuteri* R2LC and *L. plantarum* DSM 9843, but not of pectin or oatbase, significantly elevated the counts of CD4 and CD8. No significant difference was found between the two *Lactobacillus* groups. Dietary administration of ED did not significantly reduce the *lamina propria* CD4 and CD8 numbers compared with Chow feeding group.

DISCUSSION

We have demonstrated that administration of MTX to rats results in a significant impairment in intestinal immune function, which seems parallel to the severe damage in intestinal mucosal structure and microecology in the same model seen in our previous study.[15] This result is consistent with other findings which have shown that total parenteral nutrition decreases the biliary sIgA level, while it also impairs the bowel mucosa nutritional status.[1,2] The marked reduction in intestinal immunological parameters in this study might relate mainly to the significant mucosal damage from the MTX cytotoxicity. It has been proven that intestinal mucosal integrity is essential for the function of the gut-associated lymphatic tissue (GALT). The perturbation of any of the components of GALT may have serious effects on the entire mucosal immune system,[1,5] which is another area to be further investigated. Furthermore, the decreased sIgA could be related to the degradation of synthesised sIgA by consequently increased proteolytic enzymes (i.e. lysosomal enzymes) in the intestinal fluid resulting from a strong inflammatory response.[23]

Ingestion of fibre free elemental diet in this study also adversely influenced sIgA level, but not T-cell

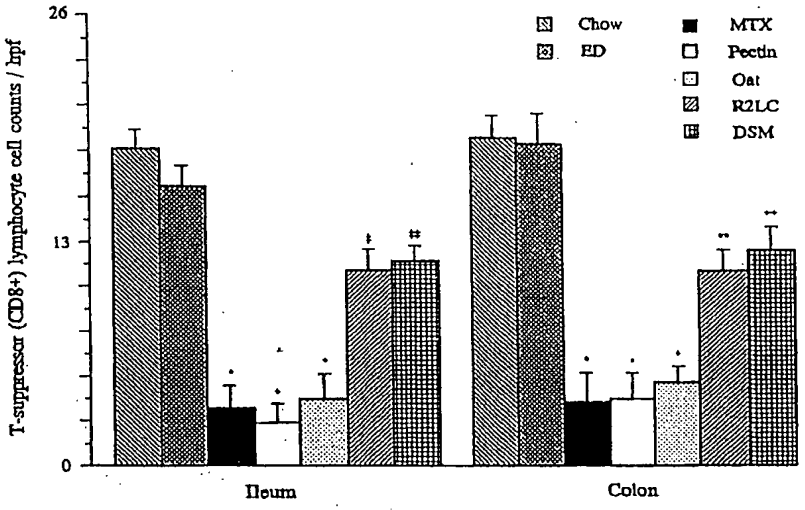

Figure 4. Different T-suppressor (CD8+) lymphocyte cell counts in the intestinal *lamina propria* from different dietary group rats received i.p. injections of either MTX (20 mg/kg) or saline. Samples were taken 72 h after the injections. Values are expressed as means ± SE. Chow and ED, groups of rats fed either Chow or elemental diet, had i.p. injection of saline. MTX, a group of rats fed elemental diet, had i.p. injection of MTX. Pectin, Oat, R2LC, DSM, groups of rats fed elemental diet with supplementation of pectin, oatmeal, *L. reuteri* R2LC, and *L. plantarum* DSM 9843, and had i.p. injection of MTX, hpf, high power field. *$P<0.001$ *vs.* Chow; $P<0.01$ *vs.* ED. **$P<0.001$ *vs.* Chow and Pectin; $P<0.05$ *vs.* ED, $P<0.01$ *vs.* MTX and Oat. $^{+}P<0.001$ *vs.* Chow and ED. $^{++}P<0.01$ *vs.* Chow and Oat; $P<0.05$ *vs.* ED; $P<0.001$ *vs.* MTX and Pectin. ‡$P<0.001$ *vs.* Chow and Oat; $P<0.05$ *vs.* ED; $P<0.01$ *vs.* MTX and Pectin. ‡‡$P<0.01$ *vs.* Chow, Pectin, and Oat; $P<0.05$ *vs.* ED; $P<0.001$ *vs.* MTX

populations. We know that the synthesis and expression of IgA in secretions appears to be sensitive to dietary alterations.[1] Under the conditions of intestinal energy and protein deficit seen in malnourishment and intestinal atrophy, the production of mucosal origin sIgA can be significantly impaired.[4] It is apparent that if GALT undergoes hypoplasia by ingestion of an elemental diet in our model, the synthesis and expression of IgA in secretions may be impaired.

The intestinal sIgA, with one fraction (insoluble) accumulated the mucosal gel and the other (soluble) spread in the intestinal lumen,[13,27] is of particular importance for its role as a primary defence line against bacterial invasion.[22,28] The reduction of intestinal sIgA may impair the intestinal protective system and allow the proliferation and overgrowth of intestinal bacteria. These mechanisms, together with the physical disruption of the mucosal barrier in our model, may lead to bacterial translocation and endotoxaemia observed in our previous investigations.[15,16]

It was indicated that supplementation of pectin and oat fibre resulted in a marked increase in intestinal mucosal mass and enhancement of mucosal structure in previous studies.[15,16] Theoretically, this might help the maintenance of GALT function and sIgA production in rats with enterocolitis. The negative results in the present study may indicate that fibres could, in some stages, improve mucosal nutritional status, but are not able to sufficiently resume the GALT function, and maintain the intestinal sIgA level in such a severe enterocolitis model. Furthermore, fibre may not be as efficient a local antigenic stimulus for generating secretory immune response as some other immunopotentiators.

Recently, various studies have focused on certain species of lactobacilli, mainly *L. casei*, *L. acidophilus*, and *L. bulgaricus* in their beneficial effects of enhancement in both systemic and mucosal immunity in the host.[22,25] Very few studies have been carried out to examine the effects of *L. plantarum* and *L. reuteri* on intestinal

immunology. We chose these two *Lactobacillus* strains in the present study, because they have previously been shown in our laboratory to have high antagonistic capacities in different models,[7,16] and superior intestinal colonisation abilities in rats[19] and humans.[11]

The enhancement of the immune response by administration of both tested *Lactobacillus* strains in this study may be due to many complex mechanisms, which could include substances produced by these organisms during the fermentation process (some metabolites, casein peptides, and bacterial enzymes).[21] It is known that casein and its peptide have beneficial effects on the GALT and the appearance of specific cell markers.[26] Certain bacteriolytic enzymes may release absorbable fragments of peptidoglycan which subsequently enhance the local and systemic immune response.[20] Moreover, lactobacilli may play a role as oral antigenic stimuli presenting to the Peyer's patch and then start the production of sIgA. In the previous study, administration of *L. plantarum* DSM 9843 and *L. reuteri* R2LC also nonspecifically enhanced the mucosal nutritional status,[15,16] which could be of great benefit to GALT function and antibody production.

This study also showed that the reduction of intestinal sIgA levels was accompanied by a decrease of CD4/CD8 populations in all enterocolitis groups, except the ED group. CD4/CD8 T cell numbers are important immunological markers, as recently shown in HIV/AIDS patients and patients on chemotherapy to evaluate their immunological functions.[6,14,24] These T lymphocytes are also involved in regulation of sIgA synthesis in mucosa *lamina propria*,[17] which may imply that MTX interfered with the host immunology by (at least one of the mechanisms) impairing the sIgA synthesis regulators, CD4/CD8 T-cell, consequently influencing sIgA production.

In summary, MTX administration resulted in a marked depletion in intestinal sIgA and gut *lamina propria* T-helper and T-suppressor cells. Administration of *L. reuteri* R2LC or *L. plantarum* DSM 9843 to enterocolitis rats improved mucosal immunity, whereas pectin or oat fibre alone did not have such effects. This indicated that the enhancement of gut immune function by administration of lactobacilli may be one of the processes through which lactobacilli protect the host from bacterial translocation and endotoxaemia in this severe enterocolitis model.

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Optimal Effect of *Lactobacillus delbrüecki* subsp. *bulgaricus*, Among Other Lactobacilli Species, on the Number of IgA and Mast Cells Associated with the Mucosa in Immunosuppressed Mice

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In previous papers we demonstrated the influence of the oral administration of different lactic acid bacteria on the systemic immune response and the protective effect against an infection with Candida albicans *in a corticoid immunosuppressed experimental model in mice. The gastrointestinal and respiratory tracts are the most common sites of entry of the infectious agents at the mucosal surfaces. The IgA secreting cells and mastocytes play an important role in host defence against pathogens. The aim of this study was to determine whether or not some* lactobacilli *strains have influence on the number of IgA and mast cells associated with the gut and bronchus mucosa, in mice immunosuppressed by corticoid therapy. Groups of animals were immunosuppressed by corticoid oral administration (50 mg kg^{-1}). They were split into four experimental groups: immunosuppressed control mice and the immunosuppressed test groups, which were given* L. casei, L. delbrüecki *subsp.* bulgaricus *or* L. acidophilus *at a concentration of 1×10^9 cells/day/mouse, on days 8 and 9 post-corticoid. We demonstrated that the* lactobacilli *strains assayed were able to increase the number of IgA secreting cells and mast cells associated with both gut and bronchus mucosa, the effect being more remarkable for* L. delbrüecki *subsp.* bulgaricus. *The enhancement of the IgA secreting cells and mast cells, in an immunosuppressed host induced by* lactobacilli *administration specially by* L. delbrüecki *subsp.* bulgaricus, *suggest the potential of its use as adjuvant therapy to protect mucosal surfaces.*

Keywords: *Lactobacilli, IgA secreting cells, mast cells, gut, bronchus, immunosuppression*

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INTRODUCTION

The glucocorticoids exert important effects on the inflammatory immune responses. They affect the growth, differentiation and function of monocytes and lymphocytes (Boumpas *et al.*, 1991), the distribution of cellular subsets, and the production of cytokines (Joyce *et al.*, 1997; Baus *et al.*, 1996). At the cellular level, glucocorticoids inhibit the access of leukocytes to inflammatory sites: interfere with the functions of leukocytes, endothelial cells, and fibroblasts; and suppress the production of humoral factors involved in the inflammatory response (Boumpas *et al.*, 1993).

Corticoids are used to suppress pathological immune responses associated with autoimmunity, inhibit rejection of allogenic tissues and to diminish inflammation associated with a wide variety of hypersensitivity disorders (Miller, 1992).

The benefits of glucocorticoid therapy can easily be offset by severe adverse side-effects; even with the greatest care, side-effects may occur such us increases of the host susceptibility to peptic ulcer, osteoporosis, avascular necrosis and atherosclerosis (Tallar-Williams & Sneller, 1994).

It has been demonstrated that lactic acid bacteria (LAB) have an immunopotentiating capacity (Gorbach, 1990; Jasui & Ohwaki, 1991; Perdigón & Alvarez, 1992; Perdigón *et al.*, 1993). *Lactobacilli* and *Streptococcus* have been suggested as potential vaccine vectors) (Iwaki *et al.*, 1990) that afford great safety, low cost and the possibility of oral administration. However these immunostimulating properties cannot be generalised for the different genera, and in some cases these characteristics are restricted to certain strains. Thus, although the use of LAB as prophylactic or therapeutic agents is an interesting field of research, their use as immunopotentiators in immunosuppression processes is still limited.

In previous work, we demonstrated that the oral administration of different species of LAB reverted the adverse effects of glucocorticoid therapy, potentiating specific and unspecific systemic immune response (Petrino *et al.*, 1995; Petrino *et al.*, 1996). As well, they induced an enhancment of IgA secreting-cells in gut associated lymphoid tissue (GALT), improving the disorders in the flora caused by antibiotic therapy. (Petrino *et al.*, 1997).

We also demonstrated that the mucosal immune response induced by some orally administrated LAB was dose dependent. At the mucosal level, the main immune mechanism developed is oral tolerance (Weiner, 1997).

We studied the influence of some *lactobacilli* species, orally administrated, on the IgA secreting cells and mast cells associated with small intestine and bronchus tissues in a mouse corticoid immunosuppression experimental model.

MATERIALS AND METHODS

Animals

BALB/c mice, each weighing 25–30 g, were obtained from a random-bred closed colony kept in our Microbiology Department. Each experimental group consisted of 20–30 mice. For each assay and days post-treatment 4–5 mice were used.

Immunosuppression Model

[illegible] phosphate, Sidus-Merck & Co. Inc.). It was tested at several doses (25, 50 and 100 mg kg^{-1}). The optimum dose was 50 mg kg^{-1} in a volume of 0.1 ml (saline), with which the immunosuppression was maintained after the 17th day post-corticoid administration.

Microorganism

The bacterial strains used were: *L. casei* CRL 431, *L. delbrüeckii* subsp. *bulgaricus* CRL 423 and *L. acidophilus* CRL 924 from CERELA Culture Collection (Tucumán, Argentina).

Lactobacilli were cultured in MRS broth (OXOID) per 8 h at 37°C. 1×10^9 cells/day/mouse were suspended in 5 ml of sterile non-fat milk (NFM 10%) and were administered at 20% v/v in the drinking water during two consecutive days, the eighth and ninth after corticoid administration. The control group received sterile milk in the drinking water under the same conditions as the test groups.

Preparation of Histological Slices

Mice were sacrificed and their small intestine and lower airways removed from both test and control group on the 3rd, 7th, 10th, 14th and 17th days post-corticoid. The samples were washed with saline solution and fixed in ice cold alcohol 96° at 2–4°C for 24 h. Then, they were dehydrated and embedded in paraffin using routine methods. Serial paraffin sections (4 μm) of the small intestine and lung of each mouse were obtained.

Histochemical Staining

Some of slices were stained with H&E and observed with a light microscope to determine any possible morphology alteration in the bronchus and gut.

In order to study the mast cells associated with gut lymphoid tissue and associated with bronchial tissue, the histological samples were stained with Alcian blue–Safranine O at pH 1 (Tas, 1977). In the gut, mast cells were counted in 30 fields. The results were expressed as number of cells/field. The cells associated with bronchial tissue were observed with a video microscope (OLYMPUS). This allowed the mast cells to be counted in the peribronchial area (three slices per mice). The results were expressed as number of cells/mm^2.

Immunofluorescence Assay

In order to count IgA producing-cells present in the bronchus and gut, direct immunofluorescence assays on histological sections were performed. The slices were immersed in three consecutive baths of xylol for 5 min each to remove paraffin. Then they were hydrated with three consecutive baths of alcohol 100° and 96° and PBS, respectively. Afterwards, the samples were incubated with α-chain monospecific anti-mouse antibody (Sigma, USA) for 30 min at room temperature. They were then rinsed with PBS and the number of fluorescent cells was counted on 30 fields (X 40 magnification) with a fluorescent microscope (Leitz). The results were expressed as number of cells/field.

Statistical Analyses

The results were expressed as the mean of n independent experiments ± standard error of the mean (SEM). The Student t-test was used to calculate the statistical significance of the results.

RESULTS

Histological Studies on Slices Stained with H&E

H&E staining was done to study possible morphology alterations of the tissues in the immunosuppressed mice. The samples did not reveal steroid-induced structural abnormalities, but a decrease in the immune cells of intestine and bronchi were observed. When the different species of *lactobacilli* were administered, we saw an enhancement of the immune cells in both tissues. The effect was more evident for *L. delbrüecki* subsp. *bulgaricus*. Figure 1(c) shows the improvement in the immune cell population of bronchus after *L. bulgaricus* administration.

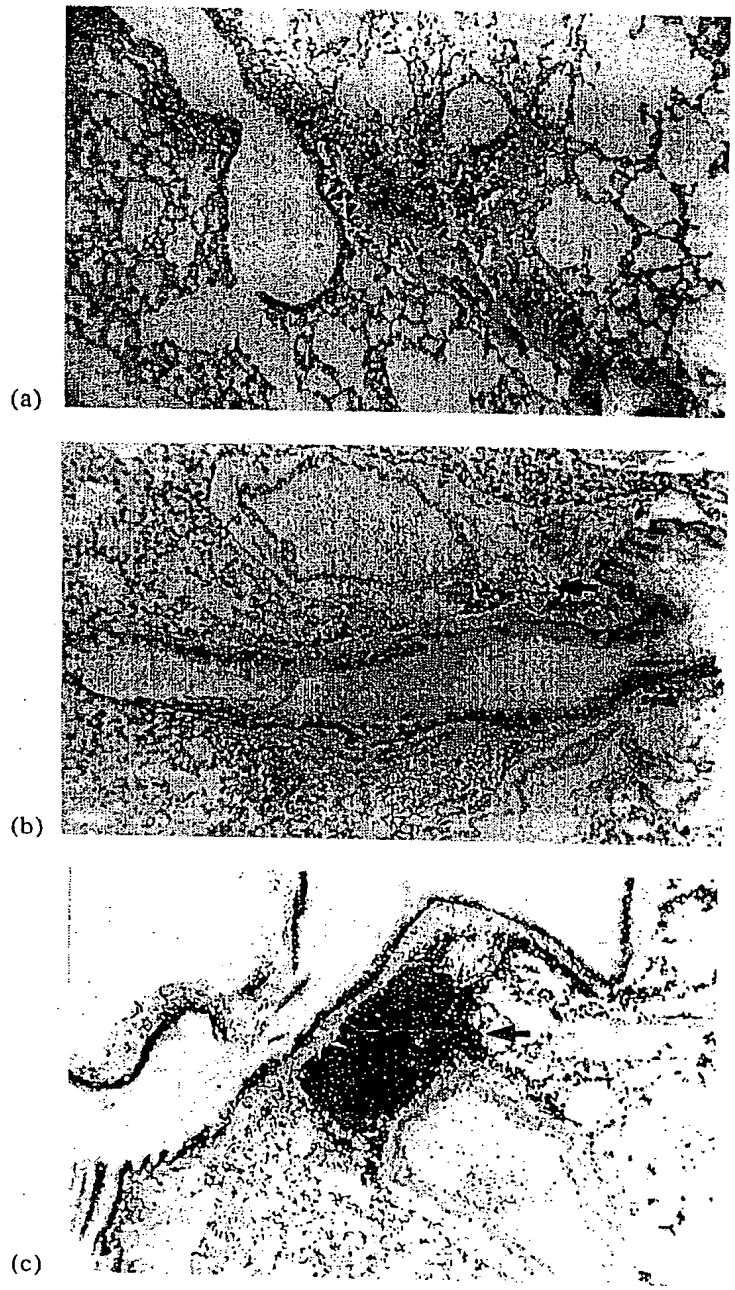

FIG. 1. Light micrograph of the bronchus associated lymphoid tissue from mice: (a) normal control; (b) immunosuppressed control; and (c) immunosuppressed and treated two days with *L. bulgaricus*. Paraffin sections were stained with H&E. Magnification × 40.

Effect of LAB Orally Administered on the Number of IgA-secreting Cells

Treatment with dexamethasone produced a remarkable decrease in the number of IgA secreting-cells, up to 10 days post-corticoid, in the small intestine (5.47 ± 2.85 cells/field *vs* control: 36.60 ± 4.89 cells/field) and in bronchus tissue (4.00 ± 2.06 cells/field *vs* control: 44.00 ± 8.16 cells/field). When the *lactobacilli* assayed were added, there was a progressive increase in the IgA secreting cells reaching normal values on day 17 in both tissues (Figure 2(a) and (b)) with significant differences with $p < 0.01$ related to the immunosuppressed control values.

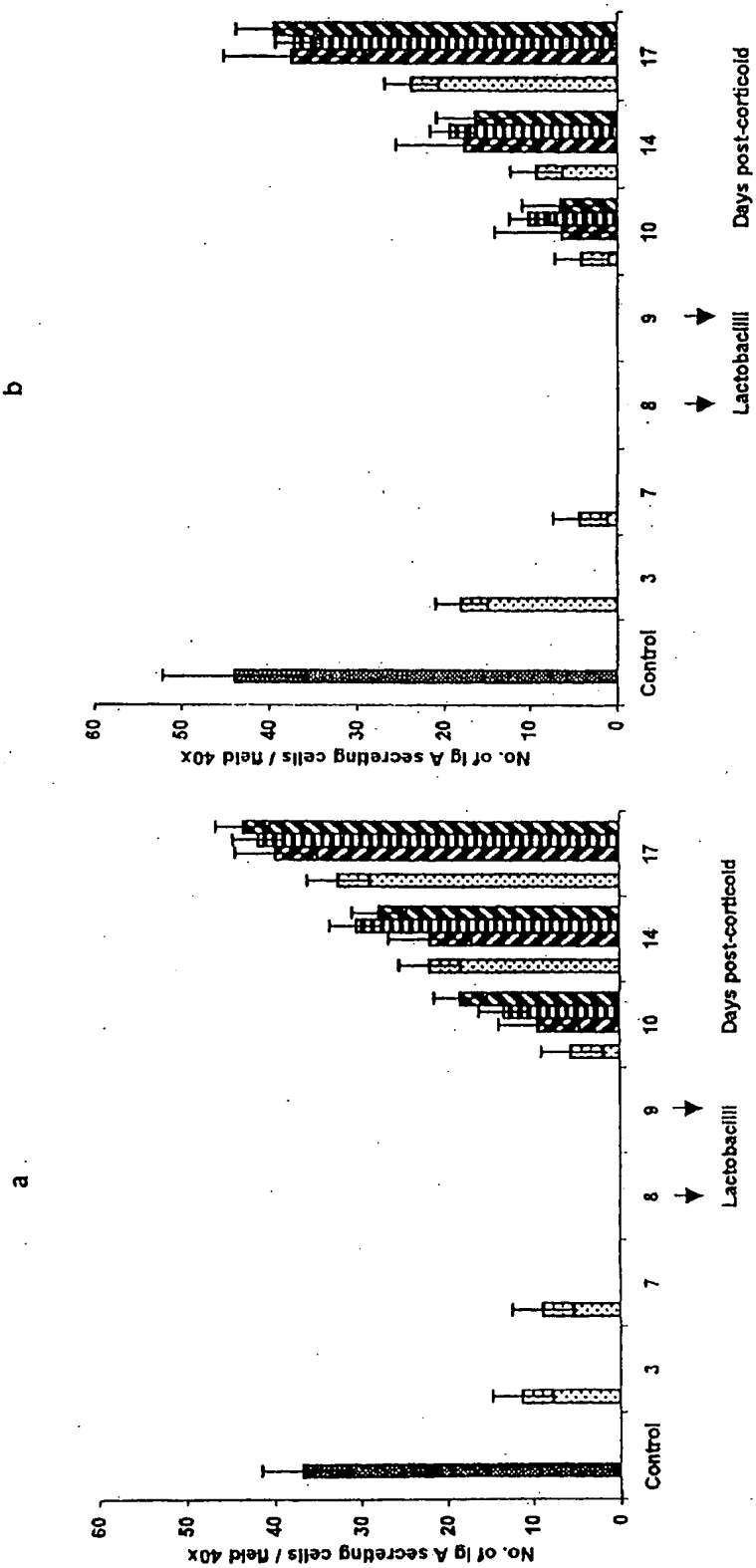

FIG. 2. Effect of *lactobacilli* on the number of IgA producing cells: (a) associated with the gut; and (b) associated with the bronchus, in a corticoid immunosuppressed mice model. IgA-secreting cells were determined by direct immunofluorescence tests on control mice, immunosuppressed by corticoid administration and immunosuppressed and treated with different *lactobacilli* strains on different days until day 17. IgA-secreting cells were counted on 30 fields (× 40 magnification). Results are the mean values ± SD, $n = 5$.

Effect of Treatment with Different LAB on Mast Cell Population in Immunosuppressed Mice

We determined that the corticoid therapy produced a great reduction in the number of mast cells associated with gut lamina propria and bronchus. When the *lactobacilli* were administered, we observed in intestine that *L. bulgaricus* induced a significant increase ($p < 0.01$) of these cells beyond the control values while, *L. casei* and *L. acidophilus* did not exert a significant effect (Figure 3(a)). When we analysed these cells associated with bronchus tissue, we saw that again *L. bulgaricus* produced an enhancement at 10 and 14 days, being at 10 days twice the control values (34.27 ± 5.78 cells/mm^2 *vs* normal values: 18.60 ± 6.84 cells/mm^2) ($p < 0.01$) Then they returned to the normal range on the 17th day post-corticoid. *L. casei* and *L. acidophilus* produced a gradual increase in the number of mast cells, reaching normal values on day 17 (Figure 3(b)).

DISCUSSION

Glucocorticoids, even when they are used for the treatment of various inflammatory and autoimmune diseases, can induce adverse effects, especially when high doses for prolonged periods of time are administered. (Spahn & Kamada, 1995). One of the most important adverse side effects of corticoid therapy is the increased susceptibility of the host to infections with opportunistic pathogens (Douglas-Briggs, 1991; Rasmussen & Arvin, 1982).

The most common sites of entry of the infectious agents are the mucosal surfaces, and the mammalian gastrointestinal and respiratory tracts are more vulnerable to infections by microorganisms. It is well known that mucosal surfaces have developed a variety of protective mechanisms, immunological and non-immunological, to protect against pathogens. Secretory immunity is the basis of specific protection at the mucosal level through IgA production (Mestecky & McGhee, 1992). The immune cells associated with mucosa have been termed MALT (mucosa-associated lymphoid tissues). The IgA-secreting cells play an important role in the immunological local defence against pathogens and damage resulting from absorption of foreign antigens.

When the mucosal immune response is stimulated, a migration of B- and T-lymphocytes is induced (Brandtzaeg *et al.*, 1991) with neutrophil recruitment, macrophage and mast cell activation providing communication between the inductor sites of Peyer's patch in the gut, with distant mucosal sites through an intermucosal circuit known as the common mucosal immune system (McDermott & Bienenstock, 1979). Based on this concept of a common mucosal immune system after antigenic stimulation of the intestinal mucosa, specific IgA precursor cell should be increased at distant mucosal sites such as the bronchus (Woloschak & Tomasi, 1983).

We demonstrated the influence of different *lactobacilli*, orally administered, in increasing the IgA secreting-cells and mast cells associated with the gut and bronchus, in a corticoid immunosuppressed host.

When we analysed the number of IgA-secreting cells, we saw that *L. delbrüecky* subsp. *bulgaricus* was more effective than *L. casei* and *L. acidophilus* in enhancing the number of IgA secreting-cells in both gut and bronchus (Figure 2(a) and (b)). All the *lactobacilli* assayed were able to reverse the immunosuppression state caused by the corticoid.

The immunodulator effect of *lactobacilli* [illegible]

observed previously, where some orally administrated *lactobacilli* species restored the number of IgA secreting cells in the small intestine after immunodeficiency caused by ampicillin (Petrino *et al.*, 1997). Dexamethasone does not induce structural abnormalities in the tissues but a diminishing in the immune cell populations as we observed in the histological slices from lung and the bronchus tissues (Figure 1(a) and (b)).

Elsewhere. Van Cott *et al.* (1994) reported that oral immunisation induced specific IgA-secreting cells in gut and bronchus and provided a complete protection against pathogens, emphasising the importance of stimulating an enhancement of these cells.

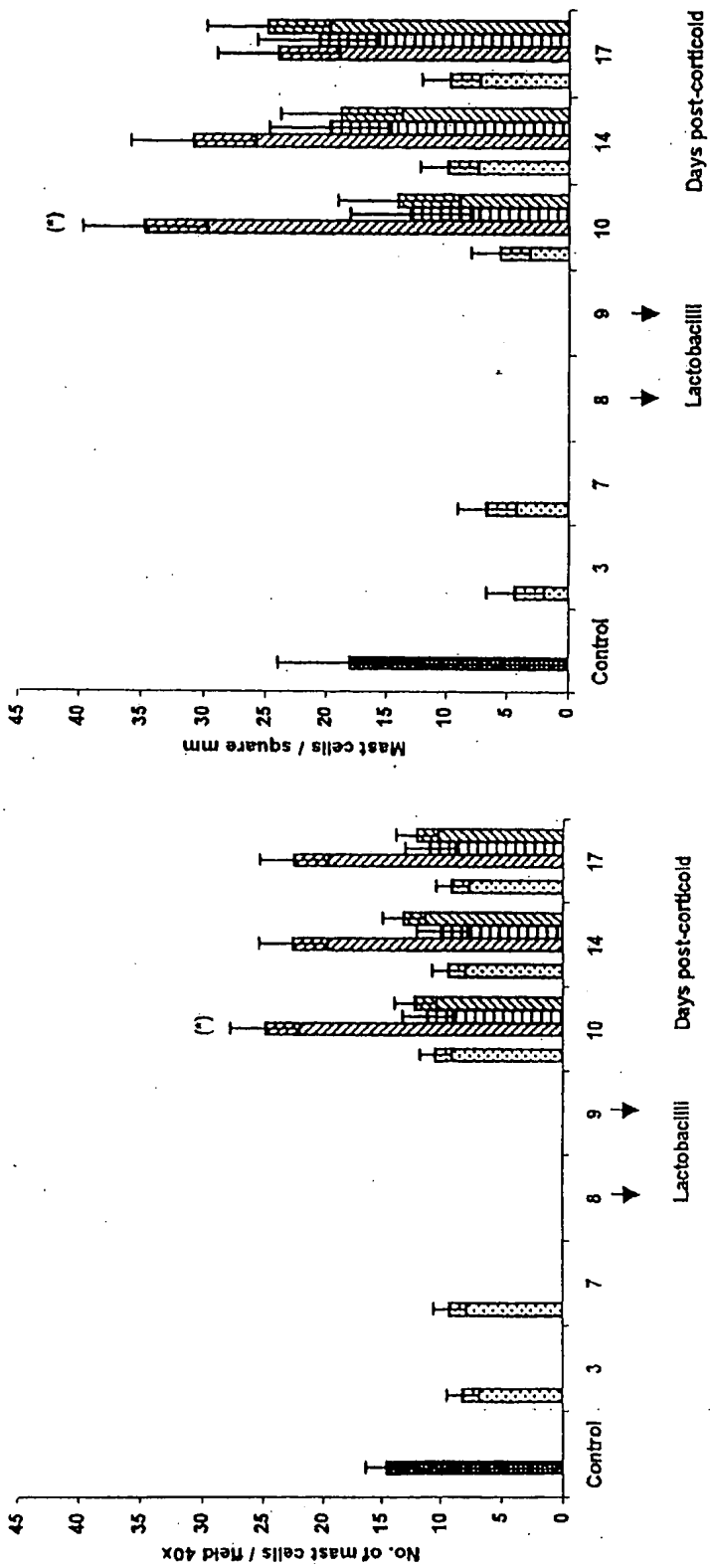

FIG. 3. Effect of different *lactobacilli* strains on the mast cells: (a) associated with the gut; and (b) associated with the bronchus in a corticoid immunosuppressed mice model. Mast cells were detected by staining with Alcian blue Safranin O on normal control, immunosuppressed and immunosuppressed and treated with *lactobacilli* strain. In the gut these cells were counted in 30 fields (× 40 magnification), and in the peribronchial area they were expressed as number of cells/mm^2. Results are the mean values ± SD, $n = 5$. *$p < 0.01$.

- Control
- Immunosuppressed
- *L. bulgaricus*
- *L.casei*
- *L.acidophilus*

Mast cells are well known as effector cells not only in allergies but also in diverse acute and chronic inflammatory diseases (Huntley, 1992). In the present study we also investigated the influence of different *lactobacilli* on these cell populations. These cells were studied considering their important role in bacteria clearance in the lung (Abraham & Malaviya, 1996) and because it has also been reported that mast cells are implicated as modulators of the increased mucosal permeability elicited by nitric oxide synthase (NOS) inhibition (Komatsu *et al.*, 1997).

Other protective roles of mast cells such as in acute bacterial peritonitis have also been reported (Hültner *et al.*, 1996). These cells are good producers of tumour necrosis factor (TNF-α) in intestinal tissue (Bischoff *et al.*, 1999) and they are able to secrete IL-5 at mucosal surfaces which can favour B1-cell differentiation to B-cell expressing IgA on its surface (Berland & Wortis, 1998).

Glucocorticoids exert profound and diverse effects on the growth, cytokine expression and granule differentiation of mouse mast cells (Eklund *et al.*, 1997), thus the increase in this population by *lactobacilli* administration would be important to improve the mucosal immunity by the cytokine that these cells can release.

We demonstrated that the effect of *L. casei* and *L. acidophilus* on the mast cells was more marked in the bronchus, while *L. bulgaricus* was active in both tissues.

The results obtained from *L. casei* and *L. acidophilus* on the intestine may be useful in reverting steroid induced immunosuppression preventing undesirable side-effects of such drugs at the intestinal level, as was shown by the counting of IgA-secreting cells and mast cells without an exacerbation of inflammatory response.

The fact that *L. bulgaricus* gave a significant increase in mast cells associated with the gut and with bronchi-associated tissue, could mean that these microorganisms could favour protection against bacterial respiratory infection. The increase observed in the IgA-secreting cells at the bronchus level by the lactobacilli assayed could mean that these LAB induced an increase in the number of IgA cells able to enter the IgA cycle, repopulating distant mucosal sites.

ACKNOWLEDGEMENTS

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Strain-dependent induction of cytokine profiles in the gut by orally administered *Lactobacillus* strains

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Abstract

Different *Lactobacillus* strains are frequently used in consumer food products. In addition, recombinant lactobacilli which contain novel expression vectors can now be used in immunotherapeutic applications such as oral vaccination strategies and in T cell tolerance induction approaches for autoimmune disease. Both for food and clinical applications of lactobacilli, proper selection of wild type strains is crucial.

For that purpose, eight different common *Lactobacillus* strains were analysed with respect to mucosal induction of pro- and anti-inflammatory cytokines, IgA-producing plasma cells in the gut, as well as systemic antibody responses against a parenterally administered antigen. Immunohistochemical analysis of cytokine-producing cells in the gut villi showed no significant induction of the cytokines IL-1α, IFN-γ, IL-4 or IL-10 after oral administration of wild type *Lactobacillus* strains. In contrast, oral administration of *L. reuteri* and *L. brevis* induced expression of the proinflammatory/Th1 cytokines TNF-α, IL-2 and/or IL-1β. Oral administration of these two strains and *L. fermentum* also significantly enhanced the IgG response against parenterally administered haptenated chicken gamma globulin (TNP–CGG). The five other strains did not show this adjuvanticity. *L. reuteri* induced relatively high levels of IgG2a compared to *L. murines*, a nonadjuving *Lactobacillus* strain.

These findings imply that different *Lactobacillus* strains induce distinct mucosal cytokine profiles and possess differential intrinsic adjuvanticity. This suggests that rational *Lactobacillus* strain selection provides a strategy to influence cytokine expression and thereby influence immune responses.

Keywords: Probiotic; Mucosal immune response; Immunohistochemistry

1. Introduction

The capability of the mucosal immune system to act in both an immunogenic as well as in a tolerogenic manner protects our body against infection as well as inadvertent inflammation. The specific functions of mucosa-lined organs, e.g. the exchange of gasses (lung) and uptake of nutrients (gut), are essential and therefore the occurrence of destructive inflammatory immune responses at those sites is undesirable. Therefore, normally T cell tolerance is induced against ingested food antigens. Nevertheless, the mucosal immune system is responsible for over 50% of the daily production of immunoglobulins [1], reflecting continuous protective immune reactions. However, ex-

Abbreviations: CGG: chicken gamma globulin; HRP: horseradish peroxidase; Mab: monoclonal antibody; TNP: trinitrophenyl; CFU: colony forming units.

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perimental induction of immune responses has proven difficult, as the precise nature of the parameters which determine the induction of such responses at the mucosa are poorly defined [2]. It is likely that local cytokine profiles may favour either Th1 type immunogenic responses or Th2 type tolerogenic responses [3].

Vaccination against infectious diseases versus peripheral T cell tolerance induction in autoimmune therapy upon oral antigen administration is thought to be at least partly dependent on particulate versus soluble nature of the antigen, respectively [4]. It has previously been shown that lactobacilli with surface-linked haptens can induce an antibody response when administered orally [5]. For oral vaccine applications recombinant lactobacilli have now been constructed which express heterologous protein on the surface of the bacteria, which serve as a particulate antigen for induction of immune responses and B cell memory [6]. Orally fed soluble autoantigens can induce peripheral T cell tolerance and thereby prevent the induction of EAE (experimental autoimmune encephalomyelitis), an animal model for multiple sclerosis [7,8]. In order to induce peripheral T cell tolerance with the use of genetically engineered lactobacilli, recombinant lactobacilli have been designed to produce and locally secrete soluble autoantigens in medium or in vivo [6].

Lactobacilli are frequently used in dairy products because of their health promoting effects such as the nonspecific enhancement of the immune response (adjuvanticity), control of intestinal infections, control of serum cholesterol levels and anti-carcinogenic activity [9]. Oral administration of these diverse species of gram-positive lactic acid bacteria with the generally regarded as safe (GRAS) status is economical and simple [10]. Since the bacterial properties required for the different applications are clearly distinct, strain selection is very important for wild type as well as recombinant lactobacilli. Therefore, in this study two properties of *Lactobacillus* strains were investigated in order to more rationally select appropriate *Lactobacillus* strains for use in oral vaccination and oral tolerance induction. First, the capability of *Lactobacillus* strains to induce specific cytokines at the mucosa by oral administration was determined in BALB/c mice, which are Th2-cell biased [11,12]. Mice were immunised i.p. with Chikungunya virus and fed eight different *Lactobacillus* strains for 4 days. Actual in vivo cytokine production was demonstrated immunohistochemically in gut tissue sections. That oral administration of an antigen can indeed lead to increased cytokine production in the gut has previously been shown with the model antigen ovalbumin (OVA) in an OVA T cell receptor transgenic mouse [3]. Here we show that oral lactobacilli can influence local cytokine production after parenteral immunisation with a pathogen (Chikungunya virus) in BALB/c mice. Second, in order to assess whether orally administered *Lactobacillus* strains were able to nonspecifically enhance the humoral response (adjuvanticity) and whether adjuvanticity correlated with the induced cytokine profiles, systemic antibody responses of mice immunised intraperitoneally with trinitrophenyl conjugated to chicken gamma globulin (TNP-CGG) were analysed.

2. Materials and methods

2.1. Bacteria

Eight *Lactobacillus* strains were used in this study. *Lactobacillus reuteri* ML1, *L. brevis* ML12, *L. gasseri* ML21 and *L. murines* CNRZ were originally isolated from mouse. Strain *L. casei* ATCC 393 was isolated from cheese and strain *L. plantarum* ATCC 14917 was isolated from sauerkraut [10]. The strains *L. fermentum* 104R and *L. plantarum* NCIB 8826 were isolated from pig and human saliva, respectively [10].

2.2. Animals and antigens

Female BALB/c mice were purchased from Charles River Laboratories (Sulzfeld, Germany). These mice were kept under filtertop hoods. The mice were 6–10 weeks old at the start of the experiments. Experiments were performed according to regulations of the Dutch law on animal experimentation. BALB/c mice are Th2-biased, favouring antibody responses [11,12].

Trinitrophenyl (TNP) was conjugated to chicken gamma globulin (CGG) (Sigma Chemical, La Jolla, CA) according to the method described by Claassen and Van Rooijen [13]. TNP–CGG was used, as a soluble protein antigen, because the dose used without adjuvant will give a suboptimal immune response. Chikungunya virus was cultured and inactivated by UV according to the method described by Nakao and Hotta [14]. This pathogen was used since it is an ARBO (arthropod borne, insect vector) virus causing disease in man and animal, which is being widely used as a model for other ARBO viruses [15].

For the detection of cytokines the following antibodies were used. Mouse antibodies against human IL-1α and IL-1β were obtained from the Instituto Ricerche Immunobiologiche Siena, Italy. The IL-2 specific rat MAb, S4B6, was a kind gift of Dr. T. Mosmann, Department of Medical Microbiology and Immunology, University of Alberta, Canada [16]. The rat MAb 11B11, directed to IL-4, was a kind gift of Dr. W.E. Paul, Laboratory of Immunology, National Institute of Allergy and Infectious Diseases, NIH, Bethesda, MD [17]. SXC-1-biotin, a rat MAb against IL-10, was obtained form Pharmingen (San Diego,

CA). The murine Mab DB-1 directed to IFN-γ and rabbit serum against TNF-α were both obtained from Dr. P.H. van der Meide from the Biomedical Primate Research Centre (BPRC), Rijswijk, Netherlands. All antibodies were crossreactive with their murine counterparts, but not with each other. Goat anti mouse-IgA–HRP (horseradish peroxidase) was obtained from KPL (Gaithersburg, MD). Streptavidin–HRP was obtained from Life Technologies (Gaithersburg, MD) and Swine anti Rabbit-Ig–HRP from Dako A/S (Glostrup, Denmark).

2.3. Oral administration of Lactobacillus strains

All strains were cultured in MRS broth (Difco, Detroit, MI) at 37°C until the OD_{690} was 1.1 (end log phase). The cells were harvested by centrifugation at 4000*g* for 20 min at 4°C, washed twice with PBS and once with 0.2 M $NaHCO_3$. Before use, the cells were resuspended in 0.2 M $NaHCO_3$ buffer (pH 8.4) to compensate for the acidic gastric environment. Each mouse received approximately 10^{10} CFU lactobacilli via the oral route. Administration was performed intragastrically.

2.4. Adjuvant activity of orally administered Lactobacillus strains

Eight different *Lactobacillus* strains were orally administered to groups of three mice for 4 consecutive days. On the first day the mice were also immunised intraperitoneally (i.p.) with 25 μg TNP–CGG in PBS. Oral administration of lactobacilli was repeated on days 21, 22, 23 and 24. The booster immunisation with TNP–CGG was performed on day 21. Serum was collected on day 7 and 14 after prime and booster immunisations. Specific antibodies against CGG and TNP were detected by ELISA.

2.5. ELISA

Polyvinyl chloride (PVC) microtiter plates (Titertek, Flow Laboratories, Irvine, UK) were coated with CGG (5 μg/ml, 50 μl/well) or TNP-BSA (5 μg/ml, 50 μl/well) overnight at 4°C. To block nonspecific antibody binding the plates were incubated for 1 h with 0.2% gelatine in PBS (50 μl/well) at room temperature. Subsequently, the plates were incubated for 1 h at 25°C with dilutions of TNP–CGG induced anti-sera and preimmune sera to correct for background reactivity. After washing, the plates were incubated with either alkaline phosphatase-labelled goat-anti mouse IgG or anti-IgM (H+L) (KPL). For the detection of CGG specific IgG1 or IgG2a antibodies, rabbit anti-mouse IgG1 or rabbit anti-mouse IgG2a antibodies (ICN Immunobiologicals, Costa Mesa, CA) were used, respectively, followed by 1 h incubation with alkaline phosphatase-labelled swine anti-rabbit Ig antibodies (Dako A/S). After addition of the substrate paranitrophenyl phosphate, the absorbance was measured at 405 nm. An IgG1 monoclonal antibody directed against CGG was used as reference for detection of IgG1 CGG specific antibodies on each ELISA-plate. A polyclonal mouse serum containing high levels of IgG2a antibodies specific for CGG was used as reference in the ELISA's to detect IgG2a specific antibodies against CGG. Sera of mice immunised with TNP–CGG in specol (water-in-oil adjuvant) served as reference serum for relative concentrations of IgG-specific antibodies against TNP and CGG.

Relative concentrations of IgG1, IgG2a and total IgG were calculated after subtraction of the absorbance of preimmune sera at the corresponding dilutions. The linear part of the reference curve was used to perform linear regression. Only those measurements of the test sera falling within the same absorbance range as reference samples used for regression (with comparable slope), were used for the calculation of the relative concentrations of IgG1, IgG2a and IgG in arbitrary units (a.u.).

2.6. Immunohistochemical detection of cytokine-producing cells in gut villi induced by lactobacilli

Eight different *Lactobacillus* strains were orally administered to groups of three mice on day 0, 2, 4 and 6, in order to obtain a high continuous level of administered lactobacilli in the gastrointestinal tract during the experiment. On day 0 the mice were also immunised i.p. with 25 μg UV-inactivated Chikungunya virus in PBS. One day after the last feeding the mice were euthanised. The first 10 cm of the small intestine containing the most Peyer's patches were taken out and rinsed in PBS. Swiss rolls were made by rolling up the small intestine around a match stick, whereby the terminal end is on the centre of the role and the "stomach" end is on the outside. Subsequently, these Swiss roles were frozen in liquid nitrogen.

Mouse cytokines were detected as described before [18]. Briefly, frozen sections (−20°C) of 8 μm were thaw-mounted on silane coated glass slides and kept overnight under high humidity at RT. The next day, slides were air-dried for 1 h and fixed at room temperature in acetone containing 0.02% H_2O_2. After air-drying for at least 10 min, sections were incubated with pre-determined optimal dilutions of primary reagents overnight at 4°C, in humidified atmosphere. Primary reagents were diluted in PBS containing 0.1% BSA. Incubations with secondary reagents diluted in PBS containing 1% BSA were done during 1 h at room temperature under high humidity. Between the

incubation steps slides were washed with PBS. Biotinylated antibodies followed by streptavidin–HRP were used for detection of IL-1α, IL-1β, IL-2, IL-4, IL-10 and IFN-γ. TNF-α was detected with swine anti-rabbit-Ig–HRP conjugate. IgA-containing plasmacells were directly detected with goat anti-mouse-IgA–HRP. Incubation with AEC (3-amino-9-ethylcarbazole) substrate for 10 min was used to reveal HRP activity (bright red precipitate) [19]. After washing, slides were counterstained with hematoxylin and embedded with glycerol/gelatin. Primary antibody/reagent omission control stainings were performed for the different reagents used.

Quantitation was performed by counting cytokine positive cells by light microscopy. Comparable areas of gut villi were scored for positive cells by two independent observers blinded to treatment. The average number of positive cells per five villi per mouse was determined. Significance of differences between all groups were determined by a single factor ANOVA. Only the significant differences of the *Lactobacillus* fed groups as compared to the $NaHCO_3$ fed group i.p. immunised with virus are indicated.

2.7. Statistical analysis

A single factor ANOVA was used to analyse the data. Since per group all mice were kept in one cage, it is allowed to use the t-test to calculate the least significant differences. When $p < 0.05$, the difference was interpreted as significant. This approach was used to compare the total IgG antibody responses, the IgG1/IgG2a ratios and the number of cytokine positive cells.

3. Results

3.1. Adjuvant activity of orally administered Lactobacillus strains

In order to examine whether there is a difference between distinct *Lactobacillus* strains in their ability to influence the humoral immune response when administered orally to BALB/c mice, which are Th2-cell biased [11,12], eight different strains were tested. Seven days after booster immunisation TNP and CGG specific IgG titres were determined in sera of mice treated according to the immunisation protocol. No differences could be detected between the levels of IgM antibodies against CGG and TNP 7 days after prime and booster immunisation. Differences in specific IgG titre were interpreted as adjuvant effects of *Lactobacillus* strains (Fig. 1). Although most of the tested strains did not show any effect as compared to the control group immunised with TNP–CGG which received $NaHCO_3$ orally, *L. reuteri* significantly enhanced the

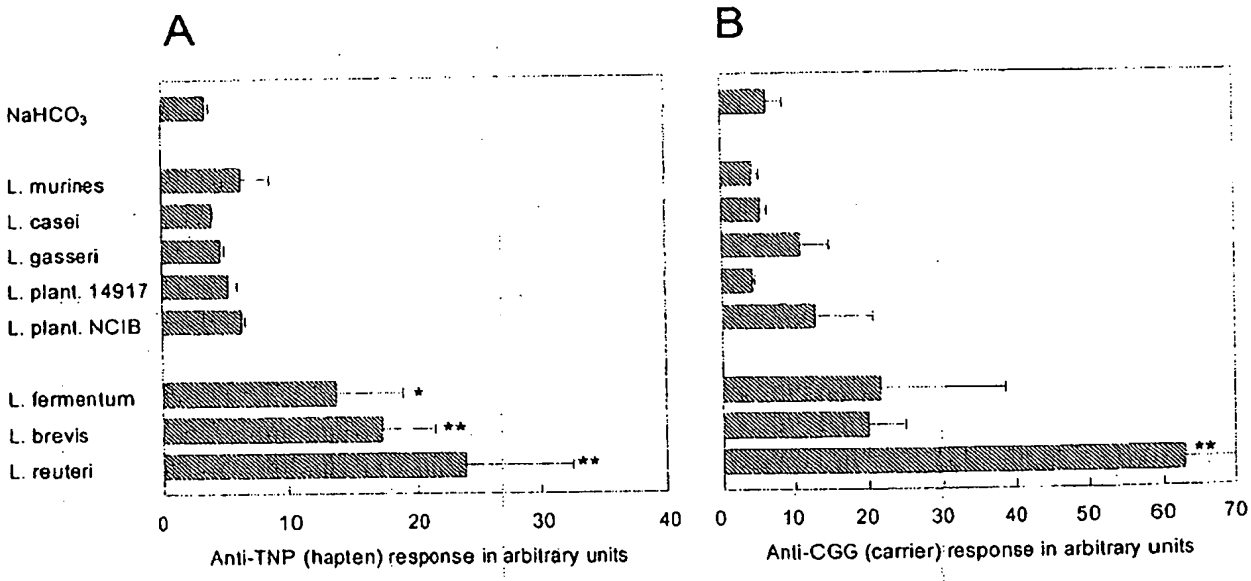

Fig. 1. Adjuvant activity of orally administered *Lactobacillus* strains. The effect of orally administered *Lactobacillus* strains on the antibody response against the thymus-dependent antigen TNP–CGG administered intraperitoneally was analysed by ELISA. The mice received a *Lactobacillus* strain or $NaHCO_3$ only orally on 4 consecutive days. On the first day the mice were also immunised i.p. with TNP–CGG in PBS. From day 21 on this protocol was repeated. The left panel shows the mean IgG antibody response ± S.E.M. against TNP per group in arbitrary units 7 days after the second immunisation. The right panel shows the IgG antibody response ± S.E.M. against the carrier protein CGG. Only significant differences compared to the $NaHCO_3$ group are indicated by asterisk. No antibody responses were detected in mice orally fed $NaHCO_3$ and immunised with PBS (data not shown). $^*p < 0.05$, $^{**}p < 0.01$, plant.: plantarum.

specific CGG antibody response ($p < 0.01$). *L. reuteri* appeared to be significantly different from all groups, except from *L. brevis* (significance not indicated in Fig. 1B). When the same sera were tested for the presence of TNP specific antibodies, again *L. reuteri* ($p < 0.01$), but also *L. brevis* ($p < 0.01$) and *L. fermentum* ($p < 0.05$) showed significant enhancement of the humoral response as compared to the TNP–CGG immunised group of mice which orally received $NaHCO_3$ only (Fig. 1A). This indicates that some orally administered *Lactobacillus* strains can, but others not, nonspecifically enhance the humoral response against a parenterally immunised antigen (adjuvanticity).

3.2. IgG1/IgG2a ratio after oral administration of Lactobacillus strains

It was demonstrated that the quantity of the antibody response evoked against the parenterally immunised antigen CGG–TNP could be enhanced by some of the orally administered *Lactobacillus* strains. Since it is generally accepted that the IgG1 response reflects activity of Th2 CD4+ T cells and IgG2a is a reflection of Th1-activity (see Ref. [20,21] and references therein), the difference in the quality of the immune response was determined by analysing those antibody isotypes. The IgG1 and IgG2a responses against CGG were detected after oral administration of 4 *Lactobacillus* strains. The antibody responses were expressed as IgG1/IgG2a ratios (Fig. 2). Only the antibody response against CGG was detected since the IgG2a responses against TNP were in most cases too low to be calculated. Oral administration of *L. reuteri* and *L. plantarum* NCIB induced relatively high IgG2a levels against CGG when compared to the control group which was fed buffer only, although the differences did not reach significance. The reduced IgG1/IgG2a ratio was significant compared to oral administration of *L. murines*, which slightly increased the IgG1/IgG2a ratio. The IgG1/IgG2a ratio induced by *L. murines* was also significantly higher than after parenteral immunisation of CGG–TNP in specol, a water-in-oil adjuvant. The ratio detected after oral administration of *L. casei* was comparable to the ratio of the control group fed buffer and the group fed *L. murines*.

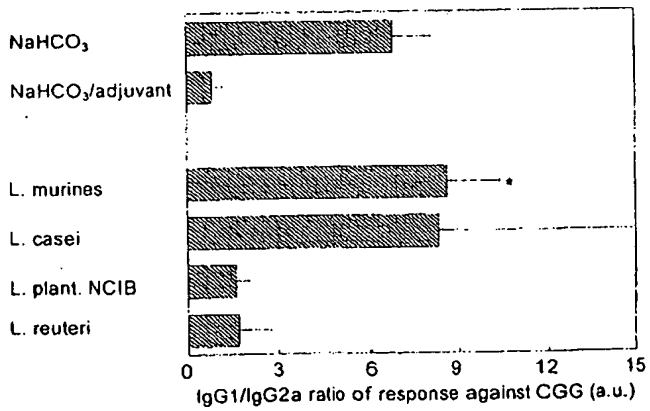

Fig. 2. IgG1/IgG2a ratio of anti-CGG response after oral administration of *Lactobacillus* strains. The effect of four orally administered *Lactobacillus* strains on the IgG1/IgG2a ratio of the response against CGG was analysed by ELISA. The mice received a *Lactobacillus* strain or $NaHCO_3$ only orally on 4 consecutive days. On the first day the mice were also immunised i.p. with TNP–CGG in PBS. A control group was fed $NaHCO_3$ and immunised i.p. with TNP–CGG in specol ($NaHCO_3$/adjuvant). From day 21 on, this protocol was repeated. Seven days after the second immunisation the IgG1 and IgG2a responses against CGG were calculated in arbitrary units with the use of reference curves. The mean IgG1/IgG2a ratio per group ± S.E.M. is shown. *$p < 0.05$ compared to the groups fed *L. plantarum* NCIB, *L. reuteri* and the group immunised with TNP–CGG in adjuvant.

3.3. Immunohistochemical detection of IgA and cytokines in gut villi

To investigate whether orally administered *Lactobacillus* strains are able to induce cytokines and IgA at the mucosa, BALB/c mice were immunised i.p. with Chikungunya virus and fed wild type lactobacilli for 4 days. Seven days after administration of the inactivated pathogen the animals were sacrificed and frozen gut tissue sections were analysed for IgA and cytokine production. In Fig. 3A the production of IgA in this primary immune response is shown in a complete cross-section of the duodenum including a Peyer's patch (lower right corner). In all animals we could find massive amounts of IgA in the B (plasma) cells located centrally in the villi (Fig. 3B). Hardly any antibody-producing B cells were seen in the Peyer's patches. As Fig. 3B shows, the lamina propria lymphocytes were the ones exclusively involved in antibody production. No IgA-containing cells were seen in the outer rim of the villi. Fig. 3B also gives a clear visual indication of the maximum number of cells per villus (8–10) that can be observed for both IgA production as well as for cytokine production by T cells (9–12, see also Fig. 3E).

The background number of positive cells was different for all cytokines, as shown in a quantitative way in Fig. 4. Fig. 3C demonstrates that for IL-2 no cytokine-producing cells are found when the animals are given $NaHCO_3$ orally and PBS intraperitoneally (no virus). It should be noted that sections on the same glass slide included positive tissue controls [22] for IL-2. Brown (not red) rims circumfering the villi were found in all samples stained for IL-2 with AEC (for revelation of horseradish peroxidase activity), even in the absence of IL-2 positive cells, representing nonspecific staining.

After intraperitoneal immunisation with Chikungunya virus, but without oral pretreatment with lactobacilli, IL-2 positive cells could be demonstrated in the lamina propria of the villi (identical to the localisation pattern of IgA) as indicated by arrows (Fig. 3D). As is clear from Fig. 3D the cells were not always homogeneously distributed over the tissue when relatively small numbers (0.2–2 on average per villus) are found.

When antigen was given in conjuction with oral lactobacilli, the number of cytokine-producing cells increased in a strain-dependent manner (Figs. 4 and 5). Most cytokine-producing cells (±10 per villus) were

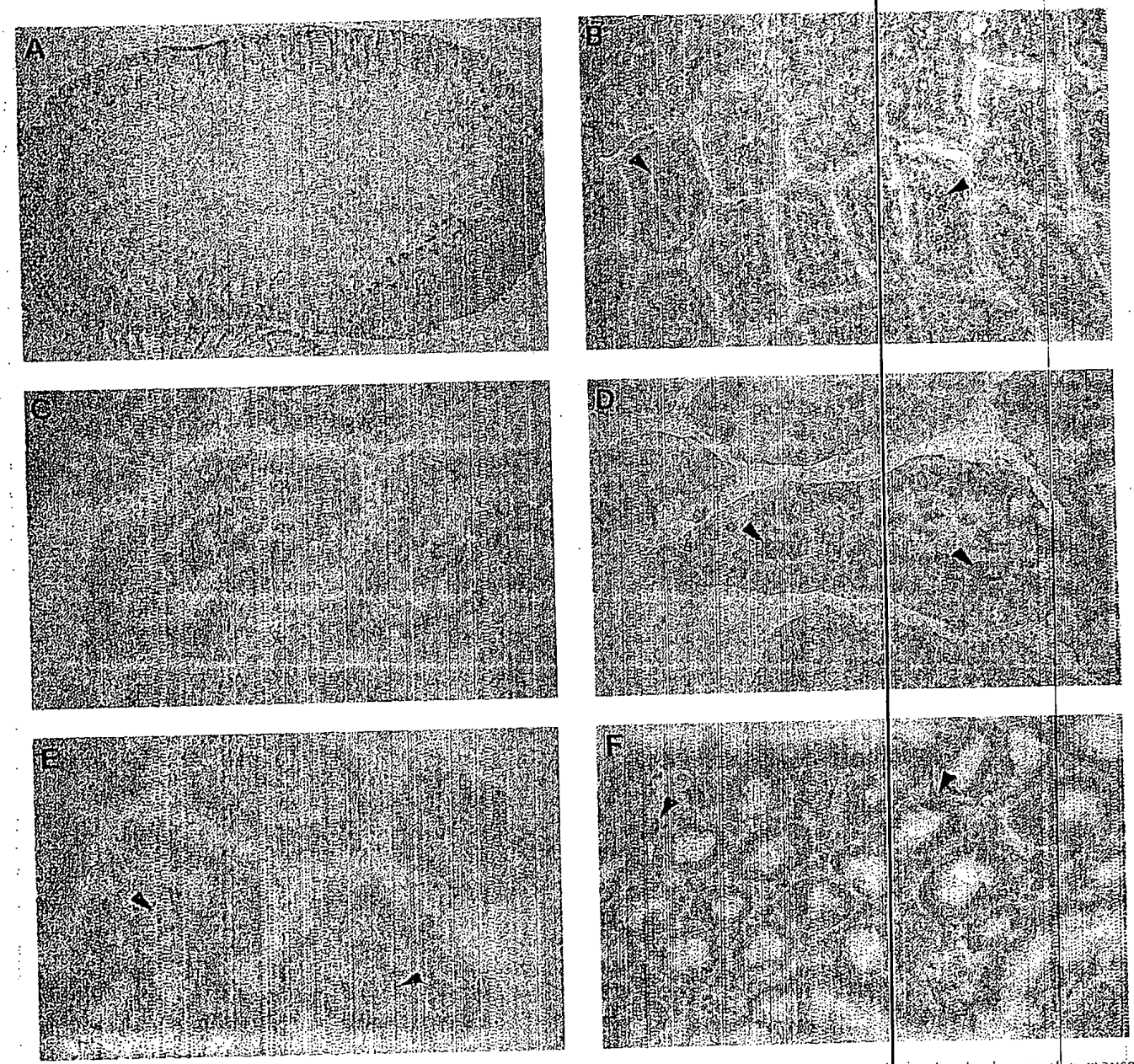

Fig. 3. Immunohistochemical detection of IgA and cytokine-producing cells in gut villi. (A) IgA-producing cells in the duodenum of a mouse immunised with Chikungunya virus. (B) Lamina propria plasma cells stained for IgA of a mouse fed with *L. reuteri* and immunised with Chikungunya virus. (C) Absence of IL-2-producing cells in gut villi stained for IL-2 of a mouse immunised with PBS and fed $NaHCO_3$. (D) IL-2-producing cells in gut villi of a mouse immunised with Chikungunya virus and fed $NaHCO_3$. (E) TNF-α-producing cells in the lamina propria of a mouse fed *L. reuteri* and immunised with Chikungunya virus. (F) Localisation of TNF-α positive cells in submucosa of mouse described in E.

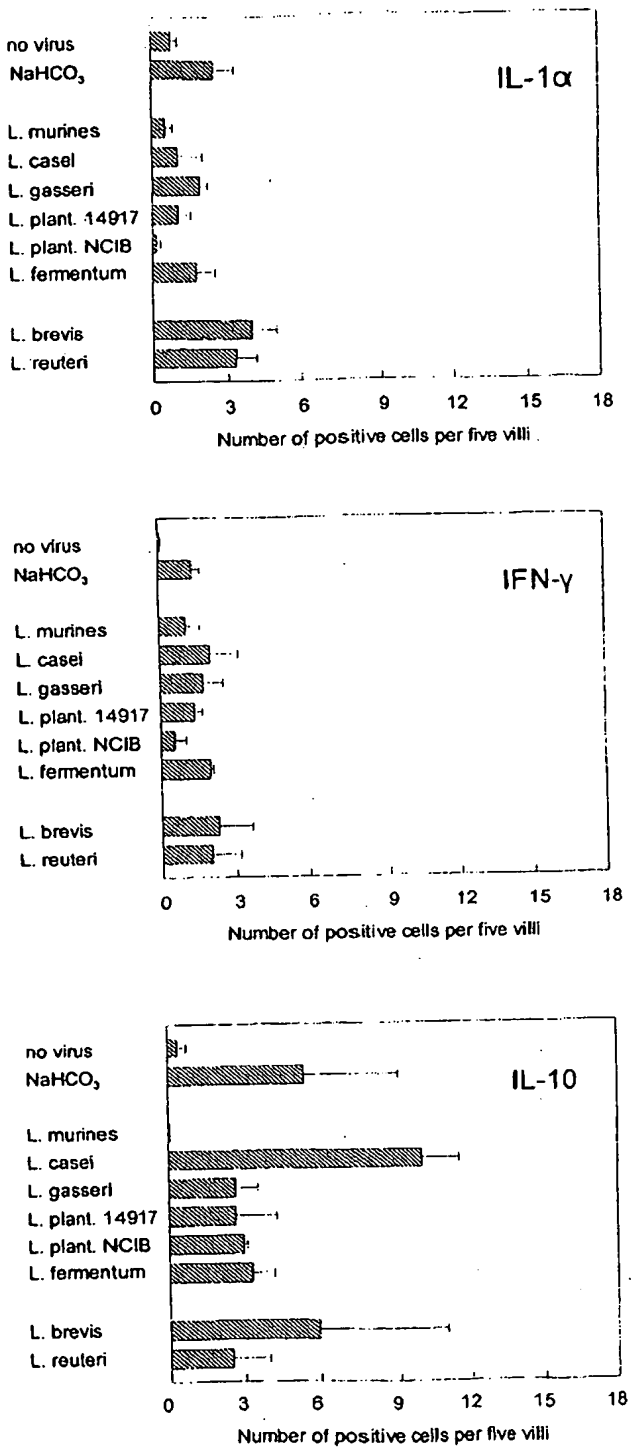

Fig. 4. Quantitative analysis of background cytokine-producing cells in gut villi. A quantitation on the immunohistochemically stained sections from mice immunised with Chikungunya virus and fed different *Lactobacillus* species was performed. Animals in the control groups were fed $NaHCO_3$ only and were immunised with TNP–CGG ($NaHCO_3$ group) or PBS (no antigen). Sections of the duodenum were stained for the cytokines IL-1α, IFN-γ and IL-10. The mean number of positive cells per five villi per group ± S.E.M. is shown.

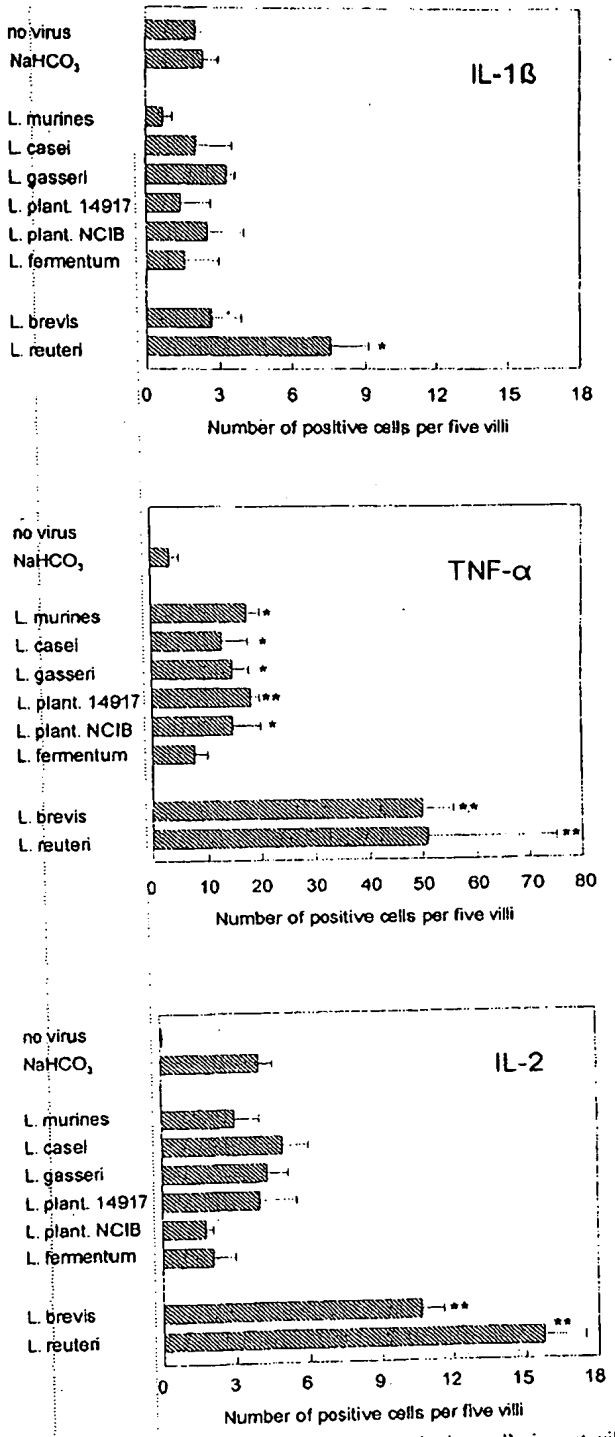

Fig. 5. Quantitative analysis of cytokine-producing cells in gut villi induced by lactobacilli. A quantitation on the immunohistochemically stained sections from mice immunised with Chikungunya virus and fed different *Lactobacillus* species was performed. Animals in the control groups were fed $NaHCO_3$ only and were immunised with TNP-CGG ($NaHCO_3$ group) or PBS (no antigen). Sections of the duodenum were stained for the cytokines IL-1β, TNF-α and IL-2. The mean number of positive cells per five villi per group ± S.E.M. is shown. Only significant differences compared to the $NaHCO_3$ fed group are indicated by asterisk. $^{*}p < 0.05$, $^{**}p < 0.01$.

found for TNF-α in *L. reuteri* treated animals as shown in Fig. 3E.

In the submucosa, where Brunner's glands are located, TNF-α was also found in the endothelial cells after oral administration of *L. reuteri* (Fig. 3F). This localisation pattern was not observed for any of the other cytokines studied.

3.4. Quantitative analysis of cytokine-producing cells in gut villi induced by lactobacilli

In order to investigate whether *Lactobacillus* strains are able to differentially induce cytokines at the gut mucosa after oral administration in Chikungunya-immunised mice, the frequency of cytokine-producing cells was determined. The levels of IgG and IgM antibodies against Chikungunya virus in sera obtained at sacrifice, were too low to be correlated to the found cytokine profiles. The cytokines IL-1α, IL-1β, IL-2, IL-4, IL-10, IFN-γ and TNF-α were immunohistochemically analysed by counting the number of cytokine-producing cells in gut villi. The background level of positive cells differed for each cytokine. Of the cytokines tested, only IL-4 was hardly detectable in all treatment groups, despite the fact that positive control tissue, (TNP-ficoll reactive spleen) contained numerous IL-4-producing cells [22,23]. The rest of the tested cytokines can be roughly divided into two groups. Some cytokines are not induced by oral administration of any of the tested *Lactobacillus* strains when compared to the control group which was immunised i.p. with virus and orally received $NaHCO_3$ only (group $NaHCO_3$) (Fig. 4). Other cytokines are induced by one or more *Lactobacillus* strains (Fig. 5). Cytokines that were not induced are IL-1α, IL-4, IFN-γ and IL-10. A low level of IFN-γ and IL-1α positive cells could be detected in all mice, but there was no significant difference with the $NaHCO_3$ fed group (Fig. 4). For IL-10 we observed that i.p. immunisation with virus only (group $NaHCO_3$) increased the frequency of IL-10-producing cells above background (no virus), although this difference did not reach significance (Fig. 4). No further enhancement by oral administration of lactobacilli was observed. Only *L. casei* tended to further increase IL-10-producing cells as compared to the virus immunised group which received $NaHCO_3$ orally (group $NaHCO_3$), but this increase was not significantly different either. The group of cytokines that can be induced by oral administration of wild type *Lactobacillus* strains consists of IL-1β, IL-2 and TNF-α (Fig. 5). An enhancement of TNF-α levels in the gut villi could be detected after oral administration of all of the tested *Lactobacillus* strains, except for *L. fermentum*, when compared to the group fed $NaHCO_3$ only (group $NaHCO_3$) (Fig. 5). *L. reuteri* and *L. brevis* both showed an approximately 15-fold increase in the number of TNF-α positive cells. Like TNF-α, IL-2 is already induced in the gut by parenteral immunisation with Chikungunya virus alone (compare group 'no virus' with group '$NaHCO_3$') (see Fig. 3D), but is significantly further increased by oral administration of *L. reuteri* and *L. brevis* (Fig. 5). *L. reuteri* is also able to induce IL-1β (Fig. 5). Clearly, there are *Lactobacillus* strains which are able to induce an inflammatory cytokine profile (*L. reuteri*, *L. brevis*).

4. Discussion

This study shows that orally administered wild type *Lactobacillus* strains can induce differential cytokine profiles in the gut of Th2-biased BALB/c mice. The *Lactobacillus* strains *L. reuteri* and *L. brevis* induce several of the proinflammatory and/or Th1 cytokines IL-1β, IL-2 and TNF-α but not anti-inflammatory Th2 cytokines such as IL-10 and IL-4 (Fig. 4). These same *Lactobacillus* strains were able to significantly enhance the systemic antibody response against a parenterally immunised antigen. In the current dogma, Th1 cells stimulate IgG2a antibodies production regulated by IFN-γ, whereas IgG1 antibodies are induced under control of the Th2 cytokine IL-4. Analysis of the antibody isotypes induced after oral administration of *L. reuteri* revealed that high IgG1 as well as high IgG2a levels could be detected. However, after oral administration of *L. reuteri* the IgG1/IgG2a ratio of antibodies directed against CGG were (significantly) lower than the IgG1/IgG2a ratios after oral administration of buffer or nonadjuving *Lactobacillus* strains. These results indicate that *L. reuteri* and probably also *L. brevis* are inducers of the cellular as well as humoral responses. Most of the tested *Lactobacillus* strains did not affect the systemic humoral immune response and showed antibody levels similar to the control group that orally received $NaHCO_3$ only. A possible explanation is that these strains act as natural gut commensals of the BALB/c mice and do not disturb the existing immune status. This is in accordance with the observation that lactobacilli are not or hardly immunogenic [24]. Possibly, the *Lactobacillus* strains that induce IL-2 and high levels of IL-1β and TNF-α and enhance the humoral immune response do evoke an immune response against themselves, leading to local inflammation. It should also be kept in mind that mucosal cytokine induction may not be directly linked to systemic antibody secretion. That there can be a relation between mucosal cytokine production and systemic antibody responses has been demonstrated by e.g. Marinaro et al. and Shi et al. [25,26].

Most cytokines are differentially regulated, which may result for instance in the presence of the Th1 cytokine IL-2 without the presence of IFN-γ, which

is also a Th1 cytokine [27]. In addition, due to different kinetics of expression of the cytokines, analysis at an other timepoint may show induction of other cytokines, such as IFN-γ. Also IL-1α and IL-1β appear to be under separate transcriptional control, which may lead to expression of one form of IL-1 without the other form [28].

Most *Lactobacillus* strains induce TNF-α, although there is a strain-dependent difference in the number of TNF-α-containing cells which is induced. This has also been seen after parenteral injection of cell wall components of *L. casei*, where *L. casei* components led to coronary arthritis due to activation of macrophages, which produced mainly IL-1 and TNF-α [29–31]. In vitro, an increased production of IL-1 and TNF-α or IFN-γ by macrophages exposed to various strains of *Lactobacillus* could be demonstrated, as well as enhanced phagocytosis [32–34]. From these studies, it seems that components in the cell wall of lactobacilli are responsible for this activation. That this is not necessary due to the peptidoglycan, a major cell wall component of gram-positive bacteria, was demonstrated by de Ambrosini et al. [35] who showed that after oral administration of peptidoglycan of four different gram-positive bacteria only the *L. casei* cell wall preparation could activate macrophages. Although no double staining was performed to determine which cells produce the detected cytokine, due to results obtained in other studies it is likely to assume that the induced TNF-α and IL-1β are produced by macrophages. Whether also IL-10 was produced by macrophages can not be excluded. TNF-α-containing cells were located in the villi like all other cytokine-producing cells. In addition to the TNF-α positive cells in the villi, TNF-α was also found to be produced by cells in the submucosa after oral administration of *L. reuteri*, but not after administration of other *Lactobacillus* strains. Also in humans with appendicitis, TNF-α can be found in the submucosa [36].

Although inflammatory/Th1 cytokine inducing *Lactobacillus* strains and non-cytokine inducing strains were identified, our panel did not contain a Th2 cytokine-inducing strain, despite the fact that a Th2-biased mouse strain was used. Only *L. casei* tended to induce IL-10 and TGF-β. Although TGF-β was clearly found to be high for *L. casei* and *L. murines*, the TGF-β data in general were not always clear to interpret and are therefore not shown. IL-10 and TGFβ have immunosuppressive effects on Th1 cells and are thought to be involved in oral tolerance [37]. An IL-10/TGF-β cytokine profile in the lamina propria can be found in ovalbumin (OVA) T cell receptor transgenic mice after oral administration of OVA, leading to T cell tolerance towards OVA [3]. From our panel of *Lactobacillus* strains, no *Lactobacillus* strain emerged, inducing a local cytokine environment permissive for oral tolerance induction.

As mentioned above, orally administered lactobacilli can activate macrophages. The known tumour-suppressive activity of lactobacilli is thought to be due to the cytotoxic action of TNF-α and IL-1 produced by macrophages [38–43]. It seems that most of the tested *Lactobacillus* strains do have this anti-tumour property, but not all. Also, many orally administered *Lactobacillus* strains, like *L. casei*, *L. reuteri*, *L. bulgaricus* and *L. acidophilus* seem able to protect against infections [44–49]. Intrinsic properties of different *Lactobacillus* strains can play a role in the effectiveness of the treatment. Therefore, the choice of the *Lactobacillus* strain is of major importance. Properties that can play a role are the capability to induce cytokines in the gut and stimulate antibody responses (adjuvanticity), but also adhesion and colonisation properties of the strains. Even the growth phase of the strain can be of importance, since we have shown that log phase versus stationary phase cultures differentially influence the IgG1/IgG2a ratio of systemic antibody responses against protein antigen-dependent on the growth phase [24].

Lactobacilli can also be used for oral vaccination and immunotherapeutic purposes in autoimmune diseases. Therefore, recombinant lactobacilli were developed which express bacterial or viral antigens on the cell surface [6]. These immunogenic particles can be used as oral vaccines. In contrast, the soluble autoantigen secreted by other recombinant lactobacilli probably will induce peripheral T cell tolerance and protect against experimental autoimmune diseases such as experimental autoimmune encephalomyelitis (EAE), an animal model for multiple sclerosis [6]. For oral tolerance induction with a recombinant *Lactobacillus* strain secreting autoantigens, a wild type strain without adjuvanticity but able to induce Th2 cytokines might be optimal. Such a strain did not emerge from the current analysed panel. On the other hand, the *Lactobacillus* strains *L. reuteri* and *L. brevis* are good candidates for the expression of immunogenic antigens for oral vaccination purposes since they may stimulate innate, cellular and humoral immune responses.

Acknowledgements

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